ILLUMINATING LATENT
BLOOD

Application methods, fixatives, alternatives and new formulas for luminol

Morgan Cheyne

ABSTRACT

Luminol is an effective chemical reagent for the detection and enhancement of latent blood and is widely utilised for this purpose throughout the forensic community. It is characterised by a pale blue chemiluminescent light which is emitted upon reaction of luminol with blood.

Many improvements to the luminol formula have been attempted over the years. In this thesis I critically evaluated three new luminol based formulas, one commonly used luminol formula and an alternative to luminol, fluorescein. These reagents were compared and contrasted in terms of sensitivity, longevity of reaction and DNA preservation as well as economic and practical considerations.

Blood pattern distortion and destruction is a major disadvantage to using luminol at crime scnenes. Five spray types and five fixatives/ shear thinning agents were evaluated on their ability to preserve the spatial morphology of bloodstain patterns on non-porous surfaces.

Lumiscene Ultra showed the highest intensity of emitted light for higher concentrations of blood. However, when blood was diluted to lower concentrations, this intensity was comparable to Bluestar Magnum, Lumiscene and Hemascein blood detecting reagents. All of the aforementioned reagents, however, had a greater sensitivity than the Grodsky formula. Hemascein had the longest reaction time with Lumiscene Ultra and Grodsky having the longest reaction times for the luminol based reagents. All of the reagents showed a certain amount of DNA degradation when compared to a water control sample. Hemascein preserved DNA to a greater extent than the rest of the reagents. Lumiscene significantly decreased the success of DNA profiling success.

The ECO spray and spray gun were found to be the best application methods for luminol for the purpose of preserving the morphology of blood patterns. The hand pump sprayer severely affected the preservation of blood patterns.

The combination of a zinc fixative, a shear thinning agent called ABA fix and the ECO spray was found to be best at fixing and preserving the morphology and spatial position of blood patterns.

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1. Introduction

Bloodshed is a common occurrence of many crimes from assault to homicide. Blood at a crime scene is important for establishing whose blood has been spilt through biological techniques such as DNA profiling and also to establish how the blood came to be in a particular pattern through reconstruction techniques (1). Consequently, the attempt to clean up or otherwise conceal blood at a crime scene by a perpetrator is also prevalent. Attempts to conceal may include painting or wallpapering over blood and removing bloodied surfaces such as mats from floors etc (1,2). Clean up attempts may include washing blood with water or a cleaning agent in an attempt to remove blood from surfaces. Garments with blood on them may be cleansed in a washing machine in an attempt to wash out the blood. Clean up attempts can greatly dilute blood resulting in blood stains which can not be seen by the unaided eye. When blood becomes invisible or near invisible to the naked eye, it is termed latent. Traces of blood that are left from 'cover-up' attempts could potentially provide valuable evidence.

Many chemicals exist for the detection and/or enhancement of blood patterns. Broadly speaking, there are two main types of chemical blood enhancement reagents. There are those which interact with blood proteins and those which are oxidised by peroxide in the presence of haemoglobin in blood, resulting in a colour change of that chemical. Some blood enhancement reagents that react with blood proteins include Amido Black, Coomassie Blue and Hungarian Red (3). These reagents react with amino acids in blood proteins to form coloured complexes. The second group includes reagents such as phenolphthalein, leukomalachite green, leucocrystal violet (LCV), luminol and fluorescein (1,3). Haemoglobin exhibits peroxidase-like activity which can catalyse the oxidation of these chemical dyes. Oxidation of these reagents in the presence of blood is accompanied by a change in colour of that reagent. Luminol is unique in that the oxidation and subsequent colour change of this reagent is accompanied by the emission of light. This emission of

light is termed chemiluminescence, whereby luminol in the presence of blood will produce photons via a chemical reaction (4).

Luminol is the favoured reagent for detecting latent blood stains as it can detect much lower dilutions of blood than other reagents, produces its own light which can be seen without aid of an alternate light source and can be applied to a large surface area easily in a relatively short time (5). Apart from the aforementioned ability to detect blood on previously washed surfaces and articles, luminol is also useful for signifying indications of mopping or cleaning, following and deciphering the direction of travel of bloody shoe prints, foot prints, blood in cracks and crevasses and drag marks (1). Following latent bloodstains could potentially elucidate or lead to other evidence. Luminol is useful for presumptively identifying bloodstains though cannot confirm the presence of blood due to the reaction of luminol with other substances resulting in a positive reaction. Another advantage luminol has due to its light emitting properties is that the colour of the substrate which a bloodstain is on does not interfere with the visualization of the reaction (4).

However, luminol is not without its disadvantages. The main disadvantage of using luminol is that the oxidised products formed when the oxidising agent in luminol reacts with blood are not insoluble. These oxidised products diffuse away from the pattern which irreversibly distorts a blood pattern (1,6). The luminol molecule reacts with these oxidised products resulting in light emission which does not truly representative of the original bloodstain. For this reason, luminol is used primarily for the *detection* of latent blood patterns, with limited interpretation (6). Bloody fingerprints are not able to be interpreted by luminol as ridge detail becomes blurred and thus unreliable. Blood patterns treated with luminol on vertical surfaces tend to "run" and blood patterns on horizontal surfaces, particularly non-porous surfaces, tend to "pool" or otherwise become distorted (1). In this thesis I present three methods to potentially reduce the destructiveness of luminol to blood stain patterns. The first is the application of these reagents to a bloodstain, the second is the use of fixatives and the third is the use of sheer thinning agents. If a method was found to reduce the destructiveness of luminol to blood patterns and prints the evidential

value of luminol as a latent blood detecting/ enhancing chemical reagent would be greatly increased.

Other disadvantages of luminol, apart from destruction of blood patterns, include having to work in the dark, difficulties with photographing the reaction, possible interference with DNA, RNA and further presumptive and confirmatory testing, potential false positives and negatives, short viewing time, health and safety issues, life of the solution is very short and reapplication can dilute and obliterate the bloodstain patterns (6,1). The sensitivity of luminol to blood, how diluted blood can be and still be detected, is one of luminols greatest advantages and improving the sensitivity is always desirable. Another reagent which can be used for the detection of latent blood stains is fluorescein. Fluorescein offers many of the advantages of luminol and also some additional advantages but also comes with some disadvantages which are discussed in a later section. Therefore also in this thesis I will be examining new formulas for luminol and an alternative to luminol, fluorescein, in an attempt to overcome some of the aforementioned disadvantages which occur with luminol and to improve sensitivity (7)

1.1 Luminol through the Ages

A good summary of the history of luminol is written by Gaensslen in the 'Sourcebook in Forensic Serology, Immunology and Biochemistry' (8). There is some debate over who synthesised luminol first. It is generally accepted that A. Schmitz of Heidelberg, Germany, was the first to synthesize the compound in 1908 (4). The chemiluminescent nature of luminol when oxidised in alkaline solution was first reported by Albrecht in 1928 (9). Albrecht experimented with a number of oxidising agents in alkaline solution in the presence of hydrogen peroxide, plant peroxidases and blood. He found that hydrogen peroxide alone only exhibited weak luminescence when compared to oxidising agents (8). In 1934, Huntress et al (10) determined a simple two-stage method for the synthesis of 3-aminophthalhydrazide from 3-nitrophthalic acid and hydrazine sulphate which he then named "luminol" which means "producer of light" (8,10,11). In 1935, Harris and Parker (12) determined the quantum efficiency for the chemiluminescence of luminol to be

about 0.3%. They also discussed the possible mechanisms for the chemiluminescence. A year later, Glueu and Pfannstiel found that the use of crystalline hemin resulted in enhanced chemiluminescence, which was confirmed in 1937 by Tamamashi (8).

The historical moment for the inclusion of luminol in forensic science practice came in 1937. Forensic scientist Specht from Germany extensively studied luminol in relation to blood detection and suggested that luminol could be photographed for analytical-legal purposes. He made two solutions; one containing luminol, calcium carbonate, hydrogen peroxide and water and the other containing luminol and sodium peroxide. Specht sprayed luminol on a variety of substances and substrates to examine specificity and versatility of luminol. He also concluded that luminol did not interfere with crystal, spectral or serological blood tests (4,8)

Proesher and Moody in 1939 investigated the reaction of luminol with blood as well as the chemical structure of luminol. They also found that chemiluminescence intensity and duration was increased with dry, old blood and that luminol could be reapplied to a bloodstain and still acquire chemiluminescence (8). Two years later, Kraul et al (1941) noted that luminol was not specific for blood and found that the maximum wavelength for chemiluminescence was at 441nm with a shift to 452nm in the presence of old blood (8). McGrath in 1942 further researched the specificity of luminol to blood by spraying luminol on a number of substances including other biological fluids. McGrath concluded that, although luminol exhibited specificity for blood and was insensitive to all the biological fluids tested, luminol should only be used as a presumptive test and conformation of blood should come from more specific serological tests (4,8)

Grodsky et al in 1951 developed a formula which could be used as a field test kit for blood detection. This formula was comprised of luminol, sodium carbonate and sodium perborate and is the formula which is most commonly used today by forensic investigators. Grodsky noted that the formula needed to be kept in a cool place away from direct light, that it showed a brief lifespan and gave a false positive reaction with copper salts (eg brass and bronze on door handles and other fixtures)

(4,8). Weber in 1966 developed a new luminol formula replacing sodium carbonate with sodium or potassium hydroxide and replacing sodium perborate with hydrogen peroxide. This formula is also used extensively in forensic science today as an alternative to the Grodsky formula (4,8).

A number of studies were conducted on the interference of luminol to other presumptive tests. Specht (1937), Proescher and Moody (1939), McGrath (1942) and Grodsky et al. (1951) all concluded that luminol gave no interference to subsequent presumptive tests (8). However, in 1971 Srch reported that luminol may interfere with the Takayama test, Lattes method for determination of ABH agglutinins and the absorption/inhibition test for ABH agglutinogens (8). Srch's findings were later confirmed by Schwerd and Birkenberger in 1977 (8). Optimizing the Photography of the luminol reaction was the objective in 1973 of Zweidinger et al. A number of films, along with camera settings and development techniques were investigated and a procedure for the photography of luminol was recommended for routine practice (8).

Over the years following the introduction of the Grodsky and Weber luminol formulas, there have been many attempts to improve luminol. Many of these improvements have been on the basis of maximising chemiluminescence intensity, minimizing interferences, making formulas less destructive to DNA, lengthening shelf life, making luminol easier to prepare in the field and to extend the lifetime of the reaction. In 2000, Dr.Loic Blum founded Bluestar® forensic, a patented luminol-based whose ingredients are a trade secret (13).Bluestar® was originally used in hunting fields for the detection of game animal blood trails and subsequently reformulated for use in forensic science as a latent blood detecting reagent (14). Following Bluestar® forensic, Bluestar® Forensic Magnum was invented claiming to be three times more sensitive than regular Bluestar® Forensic (15). Recently a new luminol formula combined with fluorescein called Lumiscene was invented by Loci Forensic Products in the Neverlands (16). This was followed this year by the invention of a more sensitive formula of lumiscene called lumiscene ULTRA (17).

Luminol has also been used in other fields of science apart from latent blood detection including high performance liquid chromatography (HPLC), immunoassay, DNA probes, DNA typing, as a substrate in western blot detection and historical and archaeological studies (9). Recent papers have also outlined the possible use of luminol, in conjunction with other methods, in determining the post mortem interval (PMI) from powdered bone samples (18,19,20).

1.2 Blood, Haemoglobin and its Derivatives

Human blood can be divided into two parts; 55% of which is a plasma component and 45% is a cellular component (22). Plasma consists of approximately 80% water and 20% solid matter, which include proteins, carbohydrates, electrolytes and vitamins (22). The cellular component of blood contains leucocytes (white blood cells), erythrocytes (red blood cells) and platelets. Leucocytes are part of the immune system and are the only cells in blood which contain DNA. Thus Leucocytes are important for forensic biology for obtaining a DNA profile. Erythrocytes are the most abundant cell type in blood and are responsible for oxygen transport in the body. They are un-nucleated which means they contain no DNA. In forensic science, erythrocytes are important for catalytic tests such as luminol and fluorescein which can utilize the peroxidase-like activity of haem and haem derivatives from erythrocytes to detect bloodstains.

There are four haem groups for every haemoglobin molecule and around 280 million haemoglobin molecules per erythrocyte (23). Haemoglobin is a metalloprotien (protein containing a metal ion) consisting of four globin protein subunits. Each of these subunits consists of a single polypeptide chain which resembles six major and 2 short alpha helices, once folded into its tertiary structure. The tertiary structure creates a hydrophobic oxygen binding pocket containing a non-protein, prosthetic haem group (24,25).

Each heam group consists of a protoporphyrin IX-Fe2+ coordination complex whereby a ferrous ion (Fe2+) is bound in the middle of the protoporphyrin IX ring by four pyrrole nitrogen atoms (27). The structures of these molecules are shown in

figure 1.1. The ferrous iron has six coordination sites so is able to bind to 6 ligands, four of which are taken by the four pyrrole nitrogens of the porphin ring. Above and below the planar ring of the porphyrin are two remaining axial coordination sites. One is occupied by a histidine on the globin polypeptide chain called the proximal histidine, while the other is occupied by either oxygen (oxygenated blood) or no ligand (deoxygenated blood) (24,4). The binding of oxygen causes the proximal histidine to move toward the porphorin ring. This moves the helix containing the proximal histidine which moves the rest of the helicies in the globon subunit. A conformational change in one subunit induces conformational changes in the other subunits resulting in an increase in affinity in haemoglobin to oxygen. This process is collectively known as the cooperativity of oxygen binding in haemoglobin (24).
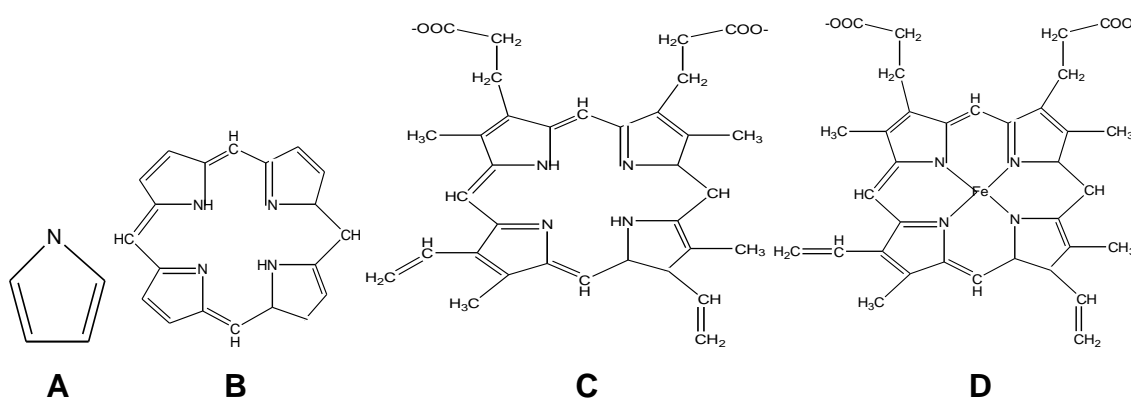
Figure 1.1 (A): Pyrrole ring (B): Porphin ring (C): Protoporphyrin IX (D) Heme

1.3 Luminol Chemistry

1.3.1 What is Luminol?

5-amino-2,3-dihydro-1,4 phthalazine-dione, simply known as 3-aminophthalhydrazide or more commonly as luminol, is a yellowish white, water soluble, crystalline powder with the chemical formula $C_8H_7N_3O_2$ arranged in the structure shown in figure 1.2.
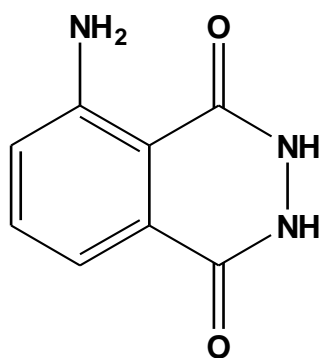
Figure 1.2: Structure of Luminol

Luminol may be synthesized beginning with 3-nitrophthalic acid. First, hydrazine (N2H4) is heated with the 3-nitrophthalic acid in a high boiling solvent such as triethylene glycol. A condensation reaction occurs, with loss of water, forming 55-nitrophthalhydrazide. Reduction of the nitro group to an amino group with sodium dithionite (Na2S2O4) produces luminol (28).

The remarkable characteristic of luminol is the pale blue chemiluminescent light produced by luminol under certain conditions (8). In water, intense chemiluminescence can be achieved by mixing luminol with a base, oxygen derivative (e.g. hydrogen peroxide) and a catalyst, either enzymatic (e.g. catalase) or mineral based (e.g. iron in blood) (22). The chemiluminescent nature of luminol makes luminol desired in many different areas of science (29). One of those areas is forensic science where by a solution containing luminol, hydrogen peroxide and a base, produces a pale blue light when sprayed onto blood which can be viewed in the dark. This solution is commonly also referred to as luminol.

1.3.2 What is Chemiluminescence?

Chemoluminescence is the emission of light resulting from a chemical reaction whereby a vibrationally excited product of an exoergic reaction relaxes to a lower energy state, usually the ground state, with the emission of photons (30,32,4). There are two main types of chemiluminescent reaction which are indirect and direct chemiluminescence (32). Luminol is an example of direct chemiluminescence which can be represented by;

A + B → [I]*→ PRODUCTS + LIGHT

Where A and B are reactants and (I)* is an excited triplet state intermediate. Excited triplet state refers to the intermediate having two unpaired electrons of the same spin whereby one electron is in a higher energy orbit (31).This excited triplet state is unstable so therefore the excess energy is rapidly lost. A slow spin-flip process converts the excited triplet state into an excited singlet state (two unpaired electrons of a different spin). The opposite spin of the electrons cancel out resulting in decay to the ground state and subsequent release of a photon (33,34,35,4). Thus light is produced without heat or excitation by any external light source, hence the colloquial name for chemiluminescent light, cold light (31). However, energy can be lost from the system by a number of radiationless processes including collisional deactivation, internal conversion, physical quenching or inter-system crossing, all of which can compete with chemiluminescence. (32,31)

Three essential energetic requirements therefore exist for there to be chemiluminescence (32,4). Firstly, there must be enough energy for a significant number of molecules to reach the electronically excited intermediate state (32,4). Secondly, the reaction is required to be exothermic, with the free energy change being in the range 170-300KJmol-1 (32). Lastly, the deactivation of the excited intermediate to ground state or lower energy state with accompanying photon emission should be energetically favourable, with competitive radiationless processes kept to a minimum (32,4).

For most analytical purposes chemiluminescence emission intensity (I_{CL}) is measured, either as a portion of the reaction or the reactions lifetime. It is a function of both the efficiency (ΦCL) and the rate of the reaction (dC/dt, molecules reacting s-1) (32).

I_{CL}= ΦCL (dC/dt)

The reaction rate depends on what species are reacting and reaction conditions. Some reactions can occur extremely slowly (>1day) while others can occur very rapidly (<1s) (32). The efficiency of the reaction (ΦCL) is the fraction of the total number of molecules reacting which emit a photon upon returning to ground state. (32,,31). This efficiency of the reaction, or chemiluminescence quantum yield, depends on three factors: Firstly, there is the fraction of reacting molecules producing an excitable molecule state, Φc, which accounts for the yield of the chemical reaction. Secondly, the fraction of reacting molecules following the correct chemical path, Φe, which relates to the efficiency of the energy transfer and lastly, the fluorescence quantum yield of the emitter, Φf, which is the fraction of these excited molecules emitting a photon upon returning to ground state. The above terms are related by the following equation: (32,31)

ΦCL = ΦcΦeΦf (31)

Different quantum yields, lifetime of reactions and wavelengths of light are emitted from different chemiluminescent reactions depending on the type of reaction and reaction conditions (4). Luminol chemiluminescence as used in forensic science is dependant on the ability of blood to catalyse hydrogen peroxide to provide the necessary oxidising agents for the reaction. The next section describes the role of the iron ion.

1.3.3 Luminol and the Iron Ion

The iron ion, which is found in heme in blood, commonly exists in two main valences; the ferrous ion (Fe^{2+}) and the ferric ion (Fe^{3+}). The ferrous ion is prone to oxidation to the ferric ion. However, in the human body, iron in its ferric state is very rare, despite oxygen binding to and subsequent dissociation from the ferrous ion. This is because there are many mechanisms, both enzymatic and non-enzymatic, to keep iron in its ferrous state (4,36). Non-enzymatically, Fe^{2+} is protected both by being encapsulated in erythrocytes and in the globin envelope. Also, erythrocytes reduced glutathione reduces Fe^{3+} to Fe^{2+}. Enzymatically, the main ways Fe^{3+} is reduced to Fe^{2+} is by NADPH-methemoglobin reductase and

NADH cytb5 reductase of the erythrocytes (4). These mechanisms are important because the ferric form of haemoglobin, methaemoglobin (MetHb), is incapable of carrying oxygen (37). In the human body, the amount of MetHb is approximately 2% of the total haemoglobin (38).

However, once blood is deposited on a surface the protective measures to prevent the oxidation of Fe^{2+} are abolished (4). Erythrocytes lyse leaving the Fe^{2+} ion exposed to the environment and enzymatic protective mechanisms are lost. As a result, spontaneous conversion of Fe^{2+} to Fe^{3+} occurs forming methaemoglobin (globin and Fe^{3+}). Further degradation causes dissociation of the haem group from the proximal histidine of the globin protein leaving haem in the Fe^{3+} state (36,4). Haem in the Fe^{3+} state can then coordinate with other ligands instead of oxygen. In alkaline conditions, coordination between the Fe^{3+} ion and the hydroxide ion (OH^-) occurs (37). The resulting structure is termed hematin which appears as an orangey-brown colour instead of the typical blood red colour (4).

Luminol contains an oxidant to help convert Fe^{2+} to Fe^{3+} and is also alkaline. Therefore the conversion of haem to hematin in bloodstains is greatly increased upon luminol application (4). Hematin is important as it catalyzes both the decomposition of peroxide and the oxidation of luminol by peroxide in a catalytic cycle (4,10).
Older bloodstains contain more hematin than newer bloodstains. That is why luminol produces a stronger reaction with aged bloodstains than fresh bloodstains. The luminol/hematin catalytic cycle is a redox reaction. Redox reactions are chemical reactions whereby the oxidation state/number of certain atoms or molecules is changed. This generally involves the loss of electrons (oxidation) by one molecule, atom or ion and a gain of electrons (reduction) by another molecule, atom or ion.

Firstly, the decomposition of hydrogen peroxide by hematin in alkaline conditions produces hydroxyl radicals (OH^{-*}) and hydroxyl anions (OH^-). Hematin ($Fe^{III}P$) is oxidised by these decomposition products in a 2 electron oxidation step forming the hydroxyl-ferryl porphyrin radical ($Fe^{IV}P^{+*}$), which is an oxidising agent. $Fe^{IV}P^+$ then

oxidises deprotonated luminol (LH-) in a 1 electron oxidation reaction to the luminol radical (L-*) and is itself reduced to hydroxyl-ferryl-porphyrin (FeIVP). FeIVP can then undergo a 1 electron reduction step to hematin by oxidising another deprotonated luminol to a luminol radical. The catalytic cycle thus restores hematin making it available for further cycling as long as there is hydrogen peroxide and luminol to utilise. That is why reapplication of luminol can be achieved with relatively no diminishment of reaction intensity. The only diminishment in reaction intensity would come from dilution of the bloodstain by the water in luminol (4)

This reaction is shown in the figure 1.3:
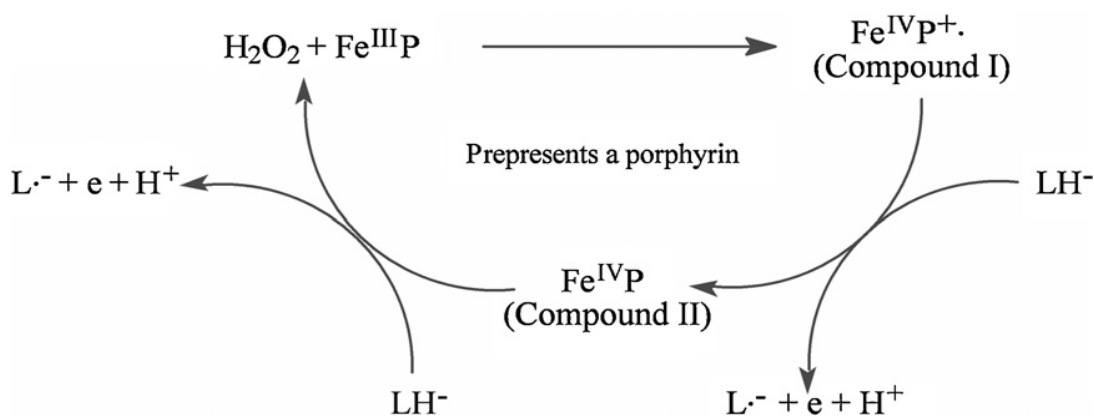
Figure 1.3: Redox cycling of the methaemoglobin iron ion in the luminol reaction producing luminol radicals.

1.3.4 The Luminol Reaction

The chemiluminescent reaction of luminol is a complex process and for many years after the discovery of luminol, it was largely unknown. In 1961, White et al researched the chemiluminescence characteristics of organic hydrazides which includes luminol and discovered 3-aminophthalate (3-APA*) was the electronically excited, photon emitting species from the luminol reaction (4). Several attempt since were made to illuminate the reaction pathway of luminol (10). During the 1980's (34,39,35), and a summary article in 1990 (32) Merenyi et al proposed the reaction

mechanism for luminol chemiluminescence that is currently accepted. This mechanism is as follows with specific reference to the Grodsky luminol formula;

The reaction starts when a base, sodium carbonate for the Grodsky's formula, is added to luminol and mixed with water. Basic conditions cause luminol to become deprotonated to its monoanionic (LH-) and dianionic (L2-) forms. Monoanionic forms are prevalent between pH 8 and 14. Grodsky luminol contains sodium perborate and Webber luminol contains hydrogen peroxide. Sodium perborated in aqueous solutions is hydrolysed to hydrogen peroxide and sodium metaborate. At low concentrations, the equilibrium shifts in favour of the hydrolysis products (40) hydrogen peroxide is decomposed by the peroxidase-like activity of hematin and its products react with hematin producing oxidising agents. These oxidising agents oxidise the deprotonated luminol in a catalytic cycle to the luminol radical (L-*) (4).

The luminol radicals (L*-) produced can react with each other to yield LH- and diazaquninone (L) or react with another one-electron oxidant to yield diazaqunione. Diazaquninone undergoes a nucleophilic attack from the hydroperoxide ion (HO2-) deriving from the deprotonation of hydrogen peroxide to form the rate-limiting, key intermediate, alpha-hydroxy-hydroperoxide LO2H2 (32). This step outlines the dependence of chemiluminescene intensity upon hydrogen peroxide concentration (7). Generally, the more hydrogen peroxide available in the reaction, the greater the chemiluminescence intensity will be.

The pathways leading to the formation of this intermediate depend on components such as overall concentrations of reactants, nature of the oxidant, additives, buffer used and pH of the reacting system (32). The decomposition of LO2H2 to ultimately yield the light emitting aminopthalate ion depends only on the pH of the system (32). At high pH values the alpha-hydroxy-hydroperoxide intermediate is further deprotonated by OH- resulting in the hydroperoxide monoanion (L-OOH) (33,32,4). Subsequent degradation of the hydroperoxide results in formation of the aminophthalate ion in a dianionic protonation state accompanied with the emission of light. While at low pH values the aminophthalate ion will retain a monoanionic protonation state which does not produce light (33,32,4). The pH of the system

should be alkaline for the hydroperoxide intermediate to be deprontonated and thus able to degrade with the emission of light. Generally, light yield increases with increasing pH with an apparent pK at pH 8.2 (30,4).

Additionally, the radical intermediate (L`-) undergoes nucleophilic attack from the superoxide radical (O2`-) via a one electron oxidation pathway (32). The super oxide radical is formed from reduction of O2 to O2`- in alkaline aqueous solution by aqueous electrons and hydrogen radicals. This also results in formation of alpha-hydroxy-hydroperoxide but is only a minor reaction because O2`- is a weaker oxidant of luminol than the hydroperoxide ion. This pathway occurs especially under conditions where the radical is in low concentration (4).

The next step is a cyclic addition of oxygen from the added hydroxide anion (OH-) to the other carbonyl carbons forming luminol endoperoxide (LO22-) (4). This is then followed by a reorganisation of bonds inside the molecule. This molecule is an oxygen adduct due to the addition of oxygen. Oxygen can exist in a triplet ground state so therefore adducts of oxygen may exist as triplet state complexes (33). Nitrogen gas leaves due to the relevant strength of its own bonds in an exothermic reaction leaving the excited state intermediate, the 3-aminophthalate dianion, in an electronically excited triplet state (33). The energy needed to form the excited triplet state is approximately 300kJmol-1 for luminol (29). A slow spin-flip process changes 3-aminophthalate from an excited triplet state to the excited singlet state which then decays to ground state with the emission of light (35,34,33).

Alpha hydroxyl hydroperoxide, apart from decomposition to yield the excited state 3-aminophthalate ion which produces light, can also decompose to yield ground-state phthalate. This pathway is known as the dark reaction and is of course, not related to the production of light. The dark reaction competes with the light reaction and results in a reduction in chemiluminescent intensity (32,4). This dark reaction occurs when the alpha hydroxyl hydroperoxide intermediate decomposes to the aminophthlate mono anion without further decomposition of the hydroperoxide to a monoanion form and therefore no subsequent endoperoxide formation (29).

The overall reaction the luminol reagent is shown below in figure 1.4.
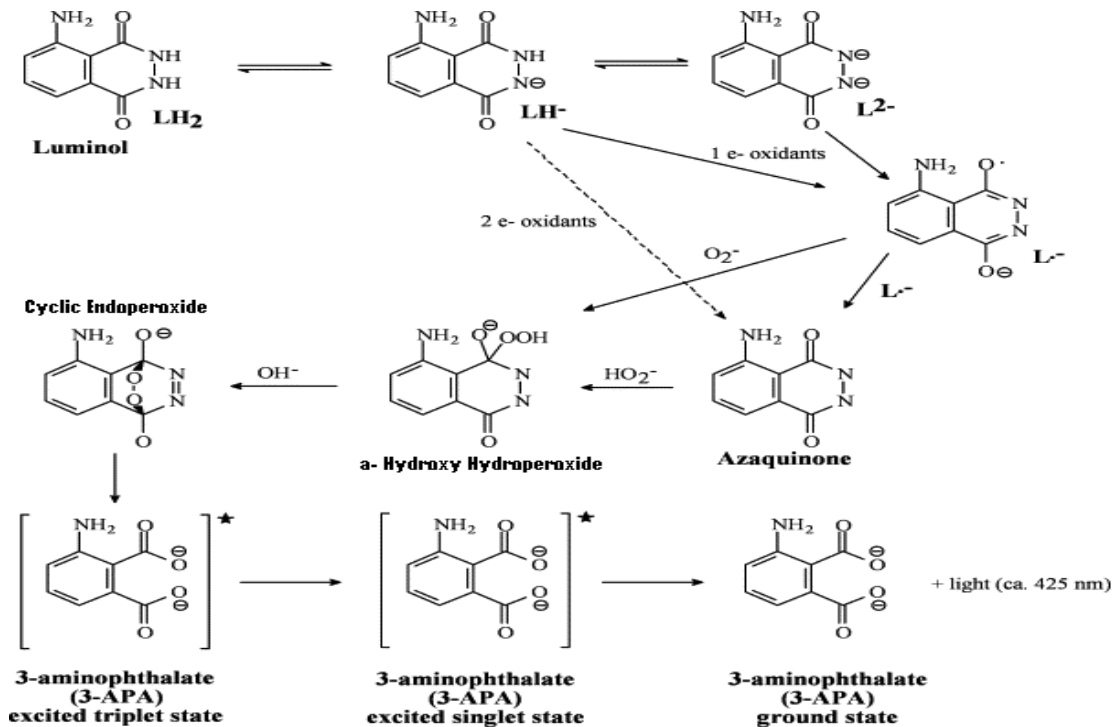
Figure 1.4: Diagram of the luminol reaction with blood- Adapted from (4)

1.4 Luminol in Forensic Science

Luminol, for forensic purposes, is predominately used to search for latent blood due to its extreme sensitivity (1). This can encompass blood which has been diluted through clean-up attempts, bloodstains left out in the rain or for following fading shoeprint trails where each step leaves less blood than the one before (1,12). Evaluation of certain blood patterns can also be achieved after enhancement with luminol, though this is limited (1,48,17,55). Luminol is commonly applied to large areas of a crime scene to quickly screen for blood. However, luminol is also used to screen smaller items for latent blood or when background colour is likely to be problematic when visualising blood with other presumptive tests (45).

Luminol is a presumptive test for blood and is therefore not entirely specific for blood meaning other substances apart from blood can also react with luminol. As such, a

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positive reaction with luminol at a crime scene does not necessarily confirm the presence of blood. Therefore, confirmatory tests such as immunological, crystal or microscope tests, or other presumptive tests such as the Kastle-Meyer test coupled with stain appearance evaluation and DNA analysis, are needed to positively identify a stain as being a blood stain after luminol application (47,13). However, if a negative result is obtained by luminol then that may preclude further investigation (52).

Luminol is usually applied as a spray in complete darkness and the light obtained from the chemiluminescence reaction can be photographed or filmed to serve as a permanent record (14,4). Once a bloodstain is located and documented, the stain can then be collected for further laboratory testing, including DNA analysis. The photographs or film taken of the luminol reaction can be presented in court as evidence (72).

1.4.1 Advantages of Luminol in the Forensic Setting

The main advantage of luminol, and why it is preferably used for the detection of latent blood, is its extreme sensitivity. Luminol is more sensitive than any other reagent commonly employed for the detection of blood (4,7). Another advantage luminol has over other chemical blood detection tests is the ease of applying luminol to a crime scene. Luminol can be sprayed around a crime scene to screen a large area for blood in a relatively short amount of time (101). The equipment needed to apply and capture the luminol reaction is relatively inexpensive. All that is required is a spray bottle and a camera (53). Also, luminol is non-corrosive and non-staining (101). This is an important quality as it does not destroy the crime scene or the items in it, including possible evidence.

Luminol can provide important information about a crime scene through blood pattern analysis as luminol can illuminate the exact position of deposited blood. Luminol can reveal patterns such as indications of cleaning, drag marks, shoe and foot impressions, including their trails (1,54). Luminol is also useful for illuminating bloodstain evidence which has been hidden, including bloodstains which have been painted over (55). Recently, studies have investigated the use of luminol, in

conjunction with other methods, in determining the post mortem interval (PMI) from powdered bone samples (71,72,73).

Importantly, luminol is a well established test for latent blood and has both general scientific and legal acceptance. Photographs or other recordings of the luminol reaction may be presented as evidence in court (54). However, the limitations of luminol should be well known and care should be taken to not overstate evidence acquired through the use of luminol. Luminol is also utilised and accepted in many other areas of science besides forensic science including molecular biology, analytical chemistry and archaeological studies (14,54).

1.4.1.1 Sensitivity

The sensitivity of luminol has been studied extensively through the years with varying results. Proescher and Moody in 1939 reported luminol reacting with blood at blood dilutions up to 1:100,000,000 if hydrochloric acid (HCl) was sprayed first to decompose haemoglobin to haemin (72). In 1953, Grodsky et al found a dilution of 1:5,000,000 (74). Lyte et al found that luminol sometimes gave a positive reaction to a complete dilution series from 1:10 to 1:1,000,000, but always down to 1:10,000 (101). Lyte et al also found that, contrary to Proescher and Moody's observations, the addition of HCl to a stain prior to luminol treatment did not increase the sensitivity of luminol but decreased it. They concluded that HCl increased background chemiluminescence which could be mistaken as a positive reaction if the blood stain was uniformly applied over a target surface (101).

Comparisons of luminol with various other blood detecting reagents have been undertaken. In 2000, Budowle et al (92) detected dilutions of 1:100,000 on non-porous substrates and usually no more than 1:100 for porous surfaces with both luminol and fluorescein. Costello (2002) experimented with cotton contaminated with bloodstains and found both luminol formulas (Grodsky and Weber) to be sensitive down to a dilution of 1:300,000. However, they found that Weber luminol produced a greater intensity and longer lasting reaction than Grodsky luminol (52). Garofano et

al (2006) furthered the work done by Budowle et al by testing the sensitivity of luminol and fluorescein at dilutions from 1:10 to 1:10,000,000 on a variety of different surface types (101). They concluded that detection depends on substrate type, agreeing with Budowle et al that the use of non-porous substrates results in lower dilutions of blood being detected. They also found that fluorescein and luminol showed different detection limits from each other on certain substrates. Quickenden et al (2006) (4) found that luminol was the most sensitive presumptive test for blood compared to Hemastix, Kastle Meyer, Leucomalachite Green and the polilight.

These variable results between studies probably originate from differences in the experimental setup. Surface type, age of blood used, amount of blood used, the amount of luminol solution applied to the stain and the concentrations of the different constituents in the luminol formula all influence the sensitivity of luminol. For example, the detection of blood at a dilution of 1:5,000,000 as described by Grodsky et al (74), which is significantly quite a lot higher than some other studies, could be due to the filter paper used in the study being soaked in diluted blood and not allowed to dry. This would lead to a higher concentration of haemoglobin than say a small amount (100ul) of dilute blood dried on filter paper and thus results in an enhanced ability to detect lower dilutions.

Another reason why this variability could exist is in the recording of reaction intensity. All of the aforementioned attempts to determine the sensitivity of luminol were undertaken in a qualitative manor. That is, the detection limit for luminol in each study was determined subjectively by the experimenter/s noting down a positive or negative reaction based on what they can see with the unaided eye. In a subjective evaluation the results rely solely on the discrimination ability of the examiner/s. Also, the longer one is in the dark, the more perceptive they are to observing the luminol reaction as their eyes adjust to the darkness (93). The following studies mentioned have attempted to quantify, or at lease semi-quantify, emitted light from the luminol reaction.

Fregeau et al (1999) tested various blood detecting reagents on bloody fingerprints diluted up to 1:200. The reactions were determined semi-quantitatively by

photographing the reaction and determining the relative strength for each reaction. A positive result was scored from 1 (poor reaction) to 5 (very strong reaction) (94). A method for the quantitative evaluation of luminol chemiluminescence intensity was developed by Quickenden et al in 2003 (92). However, the study focused on the intensity of luminol reactions with different interfering substances rather than sensitivity of luminol itself. An EMI 9635 QAM photomultiplier tube was used as the light detector. The mean peak intensity of light from each reaction of luminol with various substances was compared to a luminol reaction with just a haemoglobin solution.

Quinones (2006) compared the sensitivity of four different luminol formulas. They were Grodsky, Weber DNA, Weber Recon and Bluestar. Sensitivity was measured quantitatively using V++ image enhancement software to determine the levels of blue light intensity that have been detected by the camera. The intensity was measured in relative units allowing a comparison between the different formulas. He also determined the reaction length for the aforementioned reagents (2).

This year, a study carried out by Bilous et al used a Bio-Rad VersaFluor fluorometer to record light emissions in Relative Fluorescence Units (RFU) (95). Grodsky luminol (modified with the addition of sodium hydroxide), Bluestar and Hemascein were compared in the intensity of a reaction with liquid blood, blood stains and burnt bloodstains. Both luminol and Bluestar produced reactions with dilutions of liquid blood up to 1:800,000, with RFUs above 100 (the light intensity which would not be visible to the naked eye). The Hemascein limit for detection for this study was 1:80,000. Luminol and Bluestar gave very strong intensities (over 20,000 RFUs) to a 1:4,000 diluted bloodstain compared to 11,635 RFUs with Hemascein. For burnt bloodstains, Hemascein surpassed Grodsky luminol intensity but not that of Bluestar after one minute. However, Hemascein did produce a greater intensity than Bluestar after three minutes (2)

Dried, decomposed and generally older blood stains elicit a more brilliant and longer lasting luminescence when treated with luminol than luminol treated fresh blood (96). The reasoning behind this is that there is more conversion of haemoglobin to

methaemoglobin in old blood than that of fresh blood (97). As stated earlier, the Fe^{3+} ion incorporated in methaemoglobin reacts with hydrogen peroxide, producing the oxidizing agents capable of oxidizing the luminol molecule. Haemoglobin is also able to catalyse the luminol reaction through redox cycling between the Fe^{2+} and Fe^{3+} ions, however, a methaemoglobin catalysed luminol reaction is 1.7 times brighter (97). A brighter reaction inevitably relates to a higher sensitivity because extremely diluted samples will appear easier to see due to more light being produced. Temperature also affects the sensitivity of luminol. Higher temperatures are also shown to increase the conversion of haemoglobin to methaemoglobin, thus producing a reaction of higher intensity (97).

Part of this thesis will focus on determining the sensitivity of Grodsky luminol, Bluestar® Magnum, Lumiscene, Lumiscene Ultra and Hemascein in a quantitative and qualitative manor. Using a camera and MATLAB software, comparisons of the different reagent's sensitivities can be undertaken based on quantitatively measuring the light emitted during part of the reaction. Qualitative comparisons can also be made by comparing digitally enhanced images of the reaction produced through MATLAB. These two methods will allow a direct comparison of the relative brightness and detection limit of each of the aforementioned reagents.

1.4.1.2 Advantages of Luminol Chemiluminescence

As the light from luminol is chemiluminescent, luminol treated blood stains do not require excitation by an external light source to view the reaction. As such, luminol offers the best contrast between blood indication colour and background than any other presumptive test for blood, regardless of background colour (49). External light sources used in fluorescent reactions add the disadvantage of extra expense and can cause background interferences due to light source fluctuation (91).

Bloodstains illuminated by luminol show *in situ* exactly where the blood is with every bright sparkle of chemiluminescence corresponding to a speck of blood (54,1). Also, due to the nature of a chemiluminescent reaction, results are immediately visible.

The reaction can also be resprayed after the initial reaction has faded with equivalent or near equivalent intensity as the first spray (89).

1.4.2 Application of luminal at the Crime Scene

The most frequent way luminol is applied to a crime scene is via a common hand pump such as those used in gardens for finely spraying insecticide or water onto plants (72). Lyte and Hedgecock in 1978 (101) experimented with electropneumatic sprayers (controlled release of a compressed gas operated electrically), Freon ® (aerosol spray) and hand-pump sprayers. They found that the hand-pump sprayer with the most satisfactory overall as they are readily available from a variety of sources, inexpensive, easily portable, requires no power supply, usable at any temperature above the freezing point of the luminol solution (~0°C) and gives control over the amount of reagent applied (101).

However, the spray is not fine enough to prevent some blood patterns from being destroyed. This is probably due to the size of the droplets which are comparatively larger than say a nebulised spray mist form a compressed gas operated sprayer. Larger droplets would increase the delivery volume of the reagent in a concentrated area (7). Light producing oxidative species produced in the reaction of luminol with blood are soluble in water and the luminol solution is principally composed of water. On vertical surfaces, gravity would cause the larger droplets to "run", along with the light producing oxidative species. This causes the blood pattern to streak down the vertical surface (93). On horizontal surfaces, the droplets tend to spread or "pool" resulting in displacement of the blood pattern (93). Nonporous surfaces add the disadvantage of not absorbing any of the reagent meaning large droplets would stay on the surface of the substrate, exaggerating the "running" or "pooling" effect. Droplets from a pump sprayer may also interfere with the interpretation of blood patterns because the chemiluminescent droplets are in a similar size range as spattered blood (7).

Also, the spray is not even, meaning some areas of the blood stain will receive more luminol than other areas. Therefore there may be risk of DNA dilution and

pattern destruction from over spraying (82). For just the detection of blood however, this sprayer is convenient and efficient but for the enhancement and preservation of blood and blood patterns, it falls short. It is recommended that vertical non-porous surfaces be sprayed with a kind of nebuliser. A nebuliser sprays the luminol as a fine mist which reduces the amount of luminol sprayed on an area thus helping protect the blood pattern (92).

In an attempt to limit the destruction of luminol to blood patterns, I will be comparing different application methods of luminol to the commonly used hand pump sprayer. The other methods I will investigate include finger pump sprayers, air pump sprayers, micro-sprayers and nitrogen powered airguns. A quantitative approach will be undertaken to assess the average droplet size of each sprayer and to determine the ability of each sprayer to resolve bloody lines.

1.4.3 Limitations of luminol in Forensic Science

For all the positive attributes of luminol, it is not without some limitations. Luminol requires near complete darkness to view the reaction. Essentially, the darker the surrounding environment in which a luminol reaction is taking place, the brighter the chemiluminescence will appear. This also applies to photography as well, which can make photographing the reaction challenging (17). However, with advancements in digital camera technology over recent years, photography of the luminol reaction is easier. Also, darkening a scene is usually possible by blocking windows or other light sources or waiting until night falls (52).

Another complication is when photographing a luminol reaction the luminol reaction is short lived, typically lasting around 30 seconds before fading (95). Therefore, photographing the reaction requires someone with specific training to capture the luminol reaction in this short time (72). This can be further complicated by particular surface types, especially non-porous, vertical surfaces whereby luminol application causes a running affect. Photographs must be taken very quickly after the application of luminol to capture an image of the illuminated blood pattern before it is obliterated (93).

An area may be resprayed and achieve similar chemiluminescence to the original reaction. However, each subsequent spray dilutes blood further and results in an increased concentration of luminol solution on the blood stain, thus increasing the potential for contamination (107). This could be detrimental to DNA and subsequent presumptive and confirmatory tests as there are some fears that luminol is destructive to DNA and blood proteins. Also, the amount of blood detected by luminol at some scenes is in such a minute amount that any reduction in the quality and quantity of DNA could be the difference between obtaining a profile or not (107).

Interpretation of the luminol reaction can be complicated by interfering substances and substrates. Luminol may react with certain substances including strong oxidizers such as metal ions, peroxidises and bleaches resulting in a false positive reaction (73) or other substances which suppress a reaction with blood resulting in a false negative (92). Substrate type can also influence how a positive luminol reaction with blood appears. Therefore, the interpretation of a luminol reaction should be undertaken by a person with experience and training in luminol application (54). Also, as mentioned previously, due to the reaction of luminol with other substances besides blood, luminol cannot confirm the presence of blood and is only used to presumptively identify blood.

Morphoanalysis of bloodstains after luminol treatment is limited due to the solubility of oxidative products produced by the reaction of luminol with blood which subsequently diffuse away from the bloodstain. Interpretation of blood patterns of high detail, particularly fingerprints and impact spatter, therefore become unreliable (48,17,1,55)

Another disadvantage of luminol is that the life of the solution once mixed is relatively short, typically several hours (74). This means that luminol must be prepared fresh for each crime scene and a certain amount of mixing of the solution must be carried out at the scene to ensure that the luminol is fresh. Old solutions of luminol tend become auto-luminescent (17). However, luminol can be taken to the scene in two premixed bottles, one containing water and oxidant and the other

containing luminol and a base in solution. Once at a crime scene these two bottles can be mixed together to provide a working solution.

There have been concerns regarding the health and safety of persons preparing and using luminol. All the ingredients utilized in the luminol formulas have adverse health effects to varying degrees. In some European countries and within the UK, luminol use is limited due to the health concerns, both of the solution itself and examining items which have been treated with luminol (84). However, Castello et al found that the degree of danger of luminol is similar to that of other presumptive tests such as tetra-methyl-benzidine, o-Tolidine, phenolphthalein and leucomalachite green. O-Tolidine and possibly tetra-methyl-benzidine, however, are known carcinogens whereas luminol is not known to be carcinogenic (52).

In this thesis I will be investigating the reaction time for each reagent, including their relative reaction intensity at specified time intervals. I will also be assessing, both experimentally and non-experimentally, the shelf life of the each reagent.

1.4.4 Effect on Further Tests

Luminol is usually the last procedure carried out a crime scene so as to not contaminate or damage potential evidence, including DNA. Therefore, any obvious bloodstains found at a crime scene are collected or otherwise sampled before luminol or any other blood enhancement reagent is applied (1,18). However, sometimes at crime scenes, luminol is applied before any testing or sampling takes place to locate latent blood that may be in the area. Because other tests are needed to confirm that the stain is blood and to analyse the bloodstain for identification purposes, it is important to know, in these circumstances, if luminol treated bloodstains will have any effect on the outcome of these tests.

This effect is especially important when considering how trace amounts or diluted blood is likely to be when recovered from such bloodstains. Therefore any degradation, even slight, could be critical for subsequent tests and DNA analysis. However it is important to remember that in some cases the amount of blood available may be so small that subsequent analysis may be impossible, regardless

of wether luminol has been applied previously or not (7). Also in such cases, blood may have not been found at all if luminol had not been applied (72).

1.4.4.1 Presumptive Tests

The general consensus in studies regarding the effect of luminol on subsequent presumptive testing is that there is no effect. Grispino (1990) found that luminol did not adversely affect subsequent colour changing presumptive tests (74), which was confirmed in 1991 by Laux (7). Laux reasoned that the results were consistent with other findings that luminol may be reapplied to a bloodstain with similar results in terms of rate and intensity (7). Gross, in 1999 also found no effect to tetramethylbenzidine or phenolphthalein (commonly known as the Kastle-Meyer test) presumptive tests (75). Interestingly, luminol also does not seem to falsely react when a blood stain is previously sprayed with fluorescein; however the reverse is not true. Fluorescein can cause a false-positive reaction if sprayed after luminol application (76).

1.4.4.2 Confirmatory Tests

Subsequent identification of a bloodstain by confirmatory tests of a luminol treated bloodstain is affected depending on the type of test performed. According to Barni et al, studies by Specht, Proesher and Moody and McGarth in the first half of the 20th century found that the application of luminol on a bloodstain had no detrimental effects on the confirmatory tests used in that time (92)

Studies since have shown that luminol does not have any adverse effect on subsequent species identification tests (4,18,33), including immuoelectrophoresis (7), Ouchterlony (105), Hemocard test (106) and detection of human gamma-globuline (79). Other confirmatory tests including the Takayama test (18,33) and the hemochormagen crystal test (7) are also not compromised by luminol. ABO blood group determination in some studies was not effected by luminol (4,18), however one study did show a noticeable effect on ABO typing (105).

A few studies have found that luminol does interfere severely with electrophoretic genetic marker analysis of blood proteins (4,18,33). One study concluded that

1.4.4.3 DNA Analysis

Many studies investigating the effect of luminol on deoxyribonucleic acid (DNA) analysis have concluded that luminol causes negligible or no degradation to DNA. Conversely, there have also been some studies stating there is DNA damage, depending on the luminol formula used on the bloodstain prior to DNA acquisition.

The first study looking into the effects of luminol on DNA was conducted by Hochmeister et al in 1991. Bloodstains treated with luminol, Benzidine, phenolphthalein, o-Tolidine and leucomalachite green were tested on their ability to obtain a sufficient quality and quantity of DNA to achieve restriction fragment length polymorphism (RFLP) patterns. They concluded that luminol and Benzidine dissolved in ethanol yielded RFLP patterns consistent with that of uncontaminated controls. Phenolphthalein produced some RFLP patterns but had a more detrimental effect to DNA than luminol and Benzidine. The other presumptive tests however failed to produce any RFLP patterns. Two luminol formulas were used in this study. One formula contained sodium perborate (Grodsky) while the other instead used hydrogen peroxide (Weber) (80).

Over the years following this study, RFLP was replaced by the polymerase chain reaction (PCR) coupled with the use of microsatellite short tandem repeats (STR). This allowed for much lesser amounts of DNA needed to obtain a sufficient profile than was needed previously for the RFLP procedure, meaning smaller blood stains could be collected for DNA analysis (92). Cresap et al in 1995 investigated the effect of luminol and Coomassie on the PCR procedure concluding that neither reagent had an adverse effect (81).

Gross et al in 1999 conducted a study investigating the ability to obtain suitable DNA for PCR based DNA typing with luminol treated bloodstains that were either unwashed or washed, investigating the effect of different cleaning methods. They also investigated the effect of three different methods for extracting DNA from

luminol treated bloodstains as well as eliminating interferences from different substrates that a bloodstain could be deposited on. The Grodsky luminol formula and whole blood were used in this study. It was concluded that luminol does not effect the typing of DNA by PCR after a bloodstain has been cleaned, including with bleach. However, substrate type, especially varnished wood, can influence the ability to obtain a typeable DNA result. The three extraction methods investigated were Organic, Organic/Centricon and Chelex. It was found that all methods provided adequate amounts of DNA for PCR analysis. However, Organic and Organic /Centricon extraction gave significantly higher yields of DNA than the Chelex extraction method (75).

Another study in 2000 investigated the ability to obtain DNA from luminol treated bloodstains using a new collection method. They found that regardless of reagent treatment, the cut-off for obtaining reliable DNA profiles using the Promega GenePrint® Powerplex™ STR system was around 60pg/ul. The alternative collection method was the Puritan™ sponge tip swab which, in comparison to the conventional cotton tip swab, was a more consistent collection medium as all luminol treated bloodstains of dilutions ranging from 80% to 5% were found to give reliable DNA results. The cotton tip swab presented inconsistent results at higher dilutions. They also concluded that the limiting factor for DNA acquisition is the quantity of blood at a crime scene

Fregeau et al in 2000 studied the effect of luminol and other presumptive tests for blood including Amido Black, Crowle's Double Stain, DFO, Hungarian Red leucomalachite green and ninhydrin on bloodstains deposited on many different porous and non-porous substrate types for the acquisition of a reliable PCR results for nine different STR systems and the Amelogenin genetic marker. All the above reagents showed no degradation to DNA and thus adequate profiles at all the STR markers tested when bloodstains were exposed to these reagents for less than 54 days (94). Tobe et al however in 2007 presented results stating that leucomalachite green destroyed DNA whereas luminol was not detrimental. They also found that phenolphthalein reduced the amount of DNA recovered. (74)

Budowle et al (2000) investigated the ability to obtain typeable results at 13 STR loci with bloodstains of different dilutions treated with luminol and fluorescein on a variety of porous and non-porous substrates. It was found that wherever sufficient DNA could be recovered, typeable results at all 13 STR loci were obtained with no evidence of DNA degradation with luminol and fluorescene treated stains as well as non-treated stains. Dilutions of up to 1:1000 could be used on some non-porous surfaces (92). Barbaro et al (2003) found that both luminol and fluorescein gave typeable DNA results whether bloodstains were unwashed or washed. However, the recovery of DNA from treated bloodstains was found to depend on substrate type, type of extraction and whether bloodstains were old or fresh. The type of extraction method recommended to acquire higher yields of DNA was the organic/centicon extraction method. Older blood samples resulted in less reliable DNA profiles, however, older blood stains which were treated with luminol and fluorescein gave comparable results as those obtained from untreated stains (106). In another comparative study (2006) involving luminol and fluorescein, it was found that it is possible to obtain a DNA profile from a blood dilution up to 1:1000, which corresponds to a DNA quantity of roughly 50pg/ul, independent of the reagent used to treat the sample. They concluded that luminol and fluorescein can both be used to obtain full DNA profiles whenever it is possible to obtain a DNA profile and that both these reagents do not have a detrimental effect on DNA (101).

In a study in 2007, full profiles were obtained from bloodstains treated with luminol, fluorescein and Bluestar® when exposed to fire. One exception to this was when bloodstains were over-sprayed with reagent in order to simulate luminol reapplication if the photographer is not prepared to capture the reaction quickly. Reapplication dilutes the bloodstain resulting in less DNA able to be extracted. Reapplication of fluorescein is not usually needed as the fluorescein reaction lasts a lot longer. Another exception to DNA recovery was if bloodstains were exposed to temperatures 800º Celsius or higher, regardless of whether a reagent was applied or not.

Last year a study concluded that the quantity and quality of DNA is the determining factor for DNA profiling success as most biological samples found at crime scenes

are exposed to varying conditions of temperature, humidity and contamination. Luminol itself was found not to be detrimental to DNA profiling using Chelex 100 for extraction and the Identifier kit for amplification (73). Another study in 2009 investigated whether a DNA profile could be obtained upon incubation of luminol with blood at different concentrations. Some of the samples resulted in a decrease in the quality of DNA acquired through factors including incubation time and storage conditions. However, these factors did not affect the detection of genetic profiles once all samples showed enough DNA for STR typing (79).

Conversely, some studies have noted that luminol does have significant detrimental effects to DNA profiling, depending on the type of luminol solution applied. Quinones in 2002 found that the Grodsky formula can have detrimental effects on subsequent DNA analysis when compared to Weber, Weber II and Bluestar (86). A study this year by Almeida et al investigated the effect of luminol (Weber formular), Luminol 16®, Bluestar ® and Benzidine on the total human DNA concentration up to 120 days after sample treatment (87). Both Weber luminol and Bluestar® use hydrogen peroxide (32,31) whereas Luminol 16® uses sodium perborate tetrahydrate (89). A control bloodstain without any reagent applied to it was used to compare the quantity of DNA acquired. They found that up to 120 days after collection, Bluestar® and Weber luminol both did not significantly affect the quantity of DNA extracted from the blood stains. Luminol 16® however showed significant DNA degradation starting at day 7. Benzidine showed considerable degradation at 48 hours after treatment. At 120 days after treatment, all reagents showed significant DNA degradation compared to the control. At each of the time intervals measured, Weber luminol and Bluestar® did not differ from each other. They concluded that genotyping must be done within thirty days after the application of luminol and although the reagents in this study degraded DNA to a certain extent, PCR was not inhibited (87). Therefore, so long as there is enough DNA available, Weber luminol and Bluestar® are less likely to prevent the ascertainment of a complete genetic profile than sodium perborate based luminol formulas.

For each reagent presented in this thesis, the amount of DNA obtained from a bloodstain treated with that reagent will be compared with non-treated samples.

Also, the ability to obtain a full DNA profile from said bloodstains will also be assessed and compared.

1.4.4.3.1 Possible Mechanism for Detrimental effects

The minor detrimental effects to confirmatory tests and DNA analysis noted in some of the studies above are not likely due to the luminol molecule itself but due to the other constituents of the luminol formula reacting with proteins or DNA (92). The luminol solution contains oxidising compounds such as perborate or peroxide and has a very high pH due to the presence of strong bases (sodium carbonate or sodium hydroxide). Oxidising agents can cause oxidative damage to proteins and to pyrimidine and purine nitrogenous bases leading to fragmentation of the DNA double helix. Alkaline conditions can lead to hydrolysis of peptide bonds in proteins and also N-glycosidic bonds between the 2-deoxyribose and the nitrogen base of DNA leading to an abasic site where phosphodiester bond on the polydeoxribosephosphate strand may undergo subsequent hydrolysis (92).

Quinones study showed that the Grodsky luminol formula caused more of a detrimental effect on DNA than Weber or Bluestar® (86). Additionally, Almeida et al found that luminol 16® had more pronounced affect on DNA than did Weber luminol or Bluestar® (87). Both the Grodsky formula and Luminol 16® contain sodium perborate while Weber and Bluestar® contain hydrogen peroxide. In vivo, humans have enzymatic mechanisms to degrade hydrogen peroxide before it reaches the nucleus. Sodium perborate slowly liberates hydrogen peroxide resulting in greater penetration of the cell and thus a higher concentration of hydrogen peroxide in the vicinity of target molecules and structures. Therefore, more hydrogen peroxide means more active oxygen species which may result in more oxidative damage to DNA and proteins (90).

1.4.4.4 RNA analysis

At present, the most commonly used tests for preliminary screening of evidence for the identification of biological stains are serology-based, presumptive tests. Such tests include the Kastle-Meyer or malachite green test for blood, the acid phosphatase test for semen and the amylase test for saliva. These tests are commonly used in a sequential manor, depending on the nature of the case, with each test using a portion of the stain. Additional tests may also be required to further presumptively identify the presence of a certain biological fluid followed by confirmatory tests or species origin tests. Therefore, the use of these tests on forensic stains is potentially time consuming and can use part of a sample that may be, due to the nature of some forensic stains, degraded and of very small size to start with (69,64).

Several studies have highlighted the usefulness of ribonucleic acid (RNA) to a forensic investigation in the identification of body fluids in biological stains (62,63,64,65,66,67,68,69,70). RNA molecules come in many different forms but they are all intrinsically involved in the synthesis of proteins or regulation of gene expression. Messenger RNA (mRNA) conveys information from DNA about proteins to ribosomes where the protein is subsequently synthesized, thus providing a template for translation (91). Some proteins produced are highly specific for a particular tissue or fluid and thus the mRNA molecules which code for these proteins are also tissue specific. SPTB has been shown to be a suitable RNA marker for blood as well as other mRNA molecules including HBA, HBB, ALAS2, CD3G, ANK1 and PBGD which could be incorporated in a RNA multiplex (101). Besides blood, specific mRNA markers have also been found for other body fluids including semen, vaginal secretions, saliva and menstrual blood, providing a possible means of confirmatory identification of biological stains.

Advantages of using RNA for the identification of body fluids over traditional methods include high sensitivity, due to the option of PCR amplification, high specificity, due to fluid specific gene expression, and confirmatory testing of all forensically relevant biological stains. RNA is also able to be co-isolated with DNA thereby reducing the amount of sample needed for analysis (92). Several co-isolation methods have been described in the literature (65,66,68). Analyzing both

RNA and DNA simultaneously also allows better identification of a number of different fluids or donors in a mixture (96). Additionally, the sample size needed for RNA sampling is very small. Typically around 1ng total RNA is needed to obtain a complete mRNA profile from blood, allowing identification to be carried out on very degraded samples (97).

Other RNA molecules including micro RNA (miRNA) and small nuclear RNA (snRNA) have also been proposed for use in forensic science (67,69,70). miRNA is involved in the regulation of gene expression and snRNA is involved in mRNA processing (97). Both of these molecules are much smaller than mRNA and so could potentially be used on excessively degraded samples as these molecules would retain their integrity better relative to mRNA (91). RNA may also be useful for other facets of forensic analysis apart from the identification of body fluids including post mortem interval (PMI) estimation, wound and stain age determination and the molecular determination of the cause and circumstances of a death (67,64).

With the advancements in research and potential utility of RNA in forensic science, the preservation of RNA in bloodstains and other biological samples may be of importance to forensic investigators. It therefore appears prudent to investigate whether luminol has an effect on the ability to obtain a RNA profile. There are no papers in the literature discussing any effect luminol may have on RNA profiling.

1.4.5 Interpretation of Blood Patterns Located by Luminol

The interpretation and analysis of blood patterns located by luminol is influenced by many factors. The physical structure and chemical composition of the substrate upon which the bloodstain is found as well as any other substances apart from blood present on the substrate all influence luminol interpretation (92). Luminol is often used for scenes which have been "cleaned" which sometimes allow indications of such cleaning to be visualised. However, there are limitations to what blood patterns luminol can illuminate, particularly those of high detail such as fingerprints and other blood patterns, particularly on vertical surfaces.

1.4.5.1 Substrate Physical Structure

Broadly speaking, substrates can be placed into two categories; porous and non-porous (92). Porous surfaces are surfaces such as fabric, carpet, non-polished wood, concrete or any surface which has sufficient void spaces in the substrate to be absorbent. Porous surfaces may also include those surfaces that show superficial absorbency such as cracks or grooves in a surface or spaces between objects (92). Non-porous substrates include vinyl, tiles, glass, metal and non-textured linoleum. These surfaces do not have voids where blood can be absorbed into the substrate and are essentially non-absorbent. Therefore, less blood is retained and is not protected from the environmental, biological agents or clean up attempts (92). This results in a lower sensitivity for bloodstains on non-porous surfaces. Also, spraying luminol on to a bloody non-porous surface can lead to blood pattern distortion. Running on vertical surfaces and pooling on horizontal surfaces occurs which may result in loss of bloodstain patterns before photographs are able to be taken (11,1). The luminol reaction on non-porous surfaces therefore complicates visualisation and photography of the bloodstain (72).

When blood is shed onto a porous surface the substrate can often retain a significant amount of blood, depending on its porosity. The retained blood acquires a certain amount of protection from the biological agents, physical and chemical environmental factors and clean up attempts. Therefore, porous surfaces may retain large quantities of reasonably preserved blood which reacts very well to the luminol test. Additionally, depending on the surface, luminol can be sprayed many times without risk of excessively diluting the stains or damaging the pattern as much as non-porous surfaces. This makes for easier visualization and photography of the bloodstain.

1.4.5.2 Interfering substances and substrates

Interfering substances can be broadly divided into two categories. There are those that reduce the reaction of luminol with blood and those which react with luminol without the presence of blood (92).

Compounds which may suppress luminol chemiluminescence include; compounds with a high affinity for a specific iron oxidation state such as cyanide or sulphide, and thus compete with luminol for that iron; compounds which prevent the oxidation of luminol such as tannins, which act as anti-oxidising species; and compounds which either act as chemiluminescence quenchers or inner-filters. A quencher is excited by 3-APA* or an excited state intermediate so therefore steals the energy which would be used for chemiluminescence. Inner-filters absorb at the emission wavelength of 3-APA* preventing light being seen. However, in a crime scene, inhibitors of chemiluminescence are uncommon, except for tannins which are widely present in wood (92).

Interfering substances which cause a false positive reaction are more problematic in the forensic setting due to the possibility of these substances being present at a crime scene. These substances can be divided into three broad categories; 1) compounds which contain peroxidise or peroxidise-like activity; 2) compounds with a high oxidizing capacity towards luminol and; 3) compounds where the action mechanism towards luminol is undefined (92).

Compounds containing peroxidise or peroxidise-like activity are the most common interferences in the forensic setting. This category includes free metal ions which are combined in inorganic substances such as rust, metal objects and soils, metal ions combined with organic components such as metal porphyrins, some complex plant and bacterial proteins and pigments and lastly, enzymes belonging to the oxidoreductases class such as horseradish-peroxidases. Of particular importance to forensic science is interference due to plant peroxidases from the pulp and juice of horseradishes, turnips, parsnips and other fibrous fruits and vegetables. These substances mimic the catalytic ability of blood to break down hydrogen peroxide (92). Horseradish, turnip and parsnip pulps particularly have comparable chemiluminescent intensities to that of human haemoglobin (1,8).

The second category includes sodium hypochlorite, potassium permanganate and iodine which are present in many household industrial chemical solutions including insecticides, cleaning agents, disinfectants or antiseptics. Most commonly encountered in the forensic setting is hypochlorite in bleach as bleach is often used to clean a crime scene in an attempt to hide evidence. These substances provide oxidising species capable of oxidising luminol which mimics the oxidising species generated by the breakdown of hydrogen peroxide by Haemin (92)

The last category encompasses a large array of different substances such as oil, varnishes, glues, carpets, sinks, automobile seats and enamel paints. The exact mechanism of chemiluminescence of these substances however is unknown due to their complex chemical composition (92).

Commonly, a subjective approach to distinguishing a positive reaction from a false positive reaction is employed by forensic investigators by evaluating the emission intensity and spatial distribution of luminol chemiluminescence. Some painted, varnished and metal surfaces or objects may react with luminol producing a fairly uniformly distributed chemiluminescence which reproduces the shape, contours and dimensions of that surface or object (11,14). Some substances may also have different emission intensities of luminol chemiluminescence compared to that of blood. For example, a reaction with metals produces twinkling, intense and short emission rather than a longer lasting and more even emission is observed with blood (72). Hypochlorite-based bleaches will commonly produce bright flashes of chemiluminescence whereas with blood, the chemiluminescence is more gradually developed (92).

Some substances may have weaker emission intensities than observed with blood. However, subjective evaluation of emission intensity and spatial distribution of luminol chemiluminescence can lead to misinterpretation. For example, an object could be totally covered in blood so that the shape, contours and dimensions of that surface or object are reproduced (72). Time averaging in long-exposure photographs can lead to two different sources of chemiluminescence seeming

identical (102). Weaker chemiluminescence may be a result of diluted blood rather than a reaction with a different substance and this is why experience is a valuable component of the forensic expert (92).

If a surface is suspected to be interfering with the luminol reaction, a part of that surface which is less likely to be exposed to blood, such as a part of the surface which is covered with an object, should be tested for background chemiluminescence (72). Additionally, further presumptive tests such as the Kastle Myer test or confirmatory tests can help differentiate a bloodstain from an interfering substrate. Alternatively, a quantitative approach to discriminating between positive and false positive results is described by Quickenden and Creamer (92) and Quickenden and Cooper (108). This approach relies on the differences in the emission spectra of luminol chemiluminescence by measuring emission wavelength and emission intensity.

Many papers have studied different ways to discriminate bleach chemiluminescence from blood chemiluminescence. Amines, such as 1,2-diaminoethane, have been proposed to inhibit the oxidative chemiluminescence without affecting the reaction with haem in blood (35,36). Amino acids such as glycine have also been proposed to inhibit oxidative chemiluminescence (38). Hypochlorite ions cause a spectral shift in the luminol chemiluminescence compared to blood which can be detected using special spectroscopic equipment (8,34). Unfortunately, this spectral shift is not great enough to produce a sufficient colour change for visual discrimination (92). It has also been suggested that a crime scene where bleach is suspected be used can be just left to for 8 hours (38) or a couple of days (35,36,37) for the hypochlorite ions in bleach to decompose and thus not interfere with the luminol test for blood.

1.4.5.3 Blood Pattern Analysis

Luminol can reveal not only the presence of blood but the distribution of a bloodstain, occasionally allowing reconstruction of some events of a crime scene (14,1). Blood patterns which resemble indications of a clean-up attempt are very

important for the interpretation of a crime scene. Some blood patterns associated with cleaning include wipe and smear marks associated with cleaning, diluted bloodstains and outlines of cleaning tools such as the pattern of the bottom of a bucket. These blood patterns may give a relatively weak chemiluminescent reaction.

Luminol is useful for the enhancement of the overall dispersion and general dimensions of a blood pattern (72). For example, Luminol is useful for detecting pooled blood that has soaked into carpet padding, into cracks in wood floors or between tiles (72). Very small, scattered blood droplets can be highlighted by luminol as individual 'sparkles' of blue light (92). Impressions of bloody shoeprints, handprints, footprints and weapons can also be visualized. The direction of bloody shoeprints as well as the direction of drag marks can be deciphered and followed which may lead to more evidence (72). A recent study concluded that Bluestar® forensic luminol and leuco crystal violet (LCV) are the best chemical reagents for the enhancement of buried, bloody shoeprint impressions for the purposes of identifying class characteristics and individualising features. Bluestar® was recommended for use on non-porous substrates, whereas LCV was recommended for porous substrates (107).

However, there are some limitations in what blood patterns can be visualised and analysed. Light sources such as with luminol chemiluminescence have boundaries that are measurable, but clear specific boundaries are unlikely to exist. Therefore, the enhancement of highly detailed blood patterns such as fingerprint ridge detail, fine shoeprint features and the measurement of an individual spatter stains impact angle using luminol becomes unreliable (1,48).

The interpretation, particularly of small blood spatters associated with beating, stabbing or shooting events which have been treated with luminol should be avoided. This is because the pump spray commonly used in the application of luminol often projects luminescent droplets in a similar size range as spattered blood (7). Another interference with the interpretation of blood patterns is that luminol can often react with active insects and their by-products, which may simulate spatter (7).

Pattern distortion is caused by the diffusion of oxidised products away from a blood stain. These oxidised products are formed when the oxidising agent in luminol reacts with blood. The oxidised products are soluble in the predominantly water based luminol formula and diffuse away from the blood pattern with the water. This can irreversibly distort a blood pattern. The luminol molecule reacts with these oxidised products resulting in light emission which is not truly representative of the original bloodstain (1,48,77).

Published attempts to reduce the deleterious effect of luminol on bloodstains are scarce. Robinson in 2007 suggested three methods including; decreasing the exposure time and thus reducing the volume of luminol solution that needs to be used, thickening the luminol reagent and fixing or otherwise stabilizing the blood stain (105). Shorter exposure times require less luminol to be sprayed because of the shorter time required for the reaction to chemiluminesence. However, this method would produce images of fainter chemiluminescence which would essentially reduce the sensitivity of the luminol reagent (105).

Robert Cheeseman suggested thickening luminol and fluorescein with a shear thinning agent such as xanthan gum (106). However, the solution may become so thick it can no longer be sprayed through the common hand pump sprayer (105). Appling the thickened luminol solution with a paint roller to illuminate and preserve blood patterns has been suggested, though this method would probably bring up questions of transfer of evidence if utilised in a crime scene (105).

The third method, which involves the use of a fixative, has been attempted with sulfosalicyclic acid, a common blood fixer in Amido Black and Hungarian Red. Sulfosalicyclic acid, however, is sprayed separately and before the enhancement reagent as it must have time to work on the blood. Given that luminol is utilised to detect latent blood stains, the bloodstains cannot be seen before luminol application so therefore it is not known where the sulfosalicyclic acid should be sprayed (105). However, it may be useful in some circumstances where a particular area is

suspected to contain blood patterns which would be helpful to the investigation if they were to be preserved (93). , Nonetheless, sulfosalicyclic acid has been known to quench luminol chemiluminescence, causing a false negative result so is not recommended as a fixative for luminol (89).

In addition to investigating different application methods of luminol to reduce the destructiveness of luminol to blood patterns, I will be also investigating the use of various fixatives and shear thinning agents. These will include an alcohol fixative of 70:30 acetone : methanol; hairspray; two zinc fixatives; one containing zinc acetate, zinc chloride and calcium acetate and the other, zinc trifluoroacetate, zinc chloride and calcium acetate; xanthan gum and a commercial xanthan gum based formula called ABA fix. Experiments will be conducted in a quantitative approach by assessing the ability of each fixative or shear thinning agent combined with luminol to resolve bloody lines and blood spots.

1.4.6 Health and Safety

Luminol is very hazardous in the case of eye contact and ingestion and is hazardous in the case of inhalation and skin contact due to skin adsorption of luminol (44,43) Luminol may also be a mutagen but there is no available quantitative data on the toxicity of luminol to humans. (44,40). However, Luminol is not dangerous, under the definition of the Council of European Communities Directive 67/548/CEE (110), and has no major ecological effects (52). Luminol has been used in the past in other fields apart from forensic science including the health industry in treating alopecia and promoting blood clotting and wound healing. Biochemical research also utilizes luminol (84)

The lethal dose for luminol in one study was reported to be >500mg/Kg in rats with increased excretion of urine and sodium and decreased arterial blood pressure. The same study showed that luminol has little potential to get across the skin and into the body or to accumulate in tissues. Additionally, luminol showed rapid metabolism and excretion from the rat's bodies which suggests limited potential for chronic toxicity in humans (111).

The two ingredients of the Grodsky formula for luminol, apart from luminol are sodium carbonate and sodium perborate. Sodium Carbonate is a skin, lung and eye irritant and is hazardous if ingested and may cause sensitisation in the case of prolonged skin contact (112). Sodium Carbonate may also be a reproductive hazard. Sodium Perborate is very hazardous in the case of ingestion and hazardous in case of skin contact, eye contact and inhalation. Prolonged exposure may result in skin burns and ulcerations, respiratory irritation and central nervous system complications (41,40). Sodium perborate may also be tetrogenic and mutagenic (84).

The two remaining ingredients of the Weber formula for luminol are sodium hydroxide and hydrogen peroxide. Hydrogen peroxide is corrosive if inhaled or ingested and may be tumorigenic, mutagenic and may cause sister chromitid exchanges (84). Additionally, hydrogen peroxide burns, is an irritant and when swallowed causes nausea, vomiting and risk of intestinal perforation. Also, hydrogen peroxide needs to be kept in temperatures less than 25ºC and kept away from flammable substances and heat sources (52). One study identified sodium hydroxide as being the most hazardous chemical in luminol. They found that after spraying a room with lumino,l the concentration of sodium hydroxide in the air took 15 minutes to fall below the recommended exposure limit and further spraying increased the time (84)

However, with all the concerns about the health and safety of the various chemicals in luminol, the risk can be minimised if certain protective protocols are followed. Personal protective clothing should be worn and procedure followed when preparing and applying luminol. Personal clothing worn during the preparation and application of luminol should include gloves, eye protection, disposable overalls and an appropriate mask (14,1,44).

Luminol can be premade in the lab before going to a crime scene and kept as two separate solutions of luminol mixed with a base and the second, sodium perborate or hydrogen peroxide. Once at the crime scene these solutions can be mixed together, avoiding the risk of powders becoming airborne at the crime scene

(14,44). Some new commercial luminol formulas avoid this risk altogether by the use of tablets which can be added to a premade solution (48,49). Other protocols which can be followed to minimize exposure include preparing luminol and spraying of small items in a fume hood, limiting the number of persons present whilst spraying to those strictly necessary and removing items from a crime scene that do not require spraying or covering immovable objects (14,44).

1.4.7 Luminol Photography

A permanent record of the luminol reaction is desirable as it can be shown as evidence in court and can be referred back to by the investigator, rather than having written descriptions of the enhanced bloodstain (101). One of the first documentations of photography of the luminol reaction with bloodstains was by Spect in 1937 and Proescher and Moody in 1939 (72). In 1973 Zweidinger et al were the first to describe a successful method for obtaining a photographic record of the luminol reaction using high speed, black and white 35mm film. They also mentioned Polaroid film which had the advantage of obtaining an instant self-developing film allowing the investigator to ensure they have obtained sufficient useful photos before leaving the scene. However, Polaroid film has no permanent negative meaning a loss of detail from any copies or enlargements made from the original photo and also development conditions cannot be changed to obtain a better photo (72).

Other conditions have been experimented with using 35mm film cameras with varying exposure times, aperture sizes, film types and developing processes depending on the conditions of the scene and the intensity of the reaction (101). Fill flash photography was developed by Gimeno and Rini in 1989 which allowed simultaneous visualisation of both chemiluminescence and the background (75). Other methods to obtain a photo of a chemiluminescent reaction while still visualizing the background to a certain extent are; a film overlay negative system, by taking one picture of the reaction in dark and another in ambient light then overlaying the negatives (71); and flash bouncing, where a flash is used during

exposure on a nearby wall to illuminate the scene without washing out the chemiluminescence (72).

Since the use of film cameras, digital cameras have been employed significantly more in crime scenes as a means of capturing the luminol reaction (72). Camera settings for digital capturing of chemiluminescence have no set rules on what settings must be used. Basically, practice should be undertaken at different settings to optimize and test camera equipment (72). However, it is recommended that exposure times are long to allow enough chemiluminescent light to reach the camera sensor, ISO settings high to increase the amplification of the signal from the camera sensor and aperture number low to increase the aperture size and thus allowing more light to enter the camera (1,51,77,50). It is also recommended that photos are captured in RAW format so that images are recorded with no alterations to the image by the camera sensor and that the white balance is set to flash to ensure the correct colour of the reaction is captured. (50,77).

At the Institute of Environmental Science & Research Limited (ESR) forensic facility in Auckland, New Zealand, the following guidelines outlined in the table 1:1 below, are given for digital camera settings for luminol photography when using Nikon D100 and D70 cameras;

Camera Setting	Value
Exposure time	30 sec
Aperture	f -4.5
White balance	Flash mode
ISO equivalent	800
File format	RAW (Nikon .nef)

Table 1.1: Guidelines for luminol photography

1.5 Fixatives and Sheer Thinning Agents

Pattern distortion is a major disadvantage of luminol for detecting blood, particularly on non-porous surfaces. As stated in section 1.4.5.3, pattern distortion is caused by the diffusion of oxidised products, formed when the oxidising agent in luminol reacts with blood, away from the bloodstain. The luminol molecule reacts with these oxidised products resulting in light emission which is not truly representative of the original bloodstain. (1,48,77) This effect renders blood pattern analysis unreliable on luminol treated bloodstains. However, the amount of luminol distortion to bloodstain is dependent on substrate type as mentioned in section 1.4.5. Porous surfaces will prevent the distortion of blood patterns better than non-porous surfaces (92). These oxidised products are soluble in the predominantly water based luminol formula and diffuse away from the blood pattern with the water. This can irreversibly distort a blood pattern (1,48,77)

One way of overcoming this disadvantage would be to apply a fixative either before or with luminol. A fixative could essentially fix the oxidised products in place so that the luminol molecule reacting with these oxidised products will *in situ* emit chemiluminescent light. Therefore, theoretically only areas were the original bloodstain was will produce chemiluminescent light upon treatment with luminol.

Another way to possibly solve this problem would be to thicken the luminol solution. This can be achieved with the use of a sheer thinning agent. A thick solution of luminol would be less likely to spread away from the bloodstain. Therefore, the soluble oxidised products, and therefore the light emitting products, would be restrained to the locality of the bloodstain.

1.5.1 Histochemical fixatives

Possibly the most extensive application of fixatives in science comes from histology where fixatives are used to fix biological specimens in place for microscopy. The most well established and commonly used fixative in histology is formaldehyde.

However, formaldehyde has some key disadvantages which make it unsuitable for use in luminol. One major disadvantage is that formaldehyde is very toxic. Luminol is sometimes sprayed all round crime scenes, so if formaldehyde was to be added to the luminol formula it would prove extremely dangerous. Apart from the obvious toxic and carcinogenic properties of formaldehyde, its mechanism of cross-linking may damage proteins (114).

Also, formaldehyde can be detrimental to DNA and RNA. Formaldehyde initially causes a reversible and rapid hydroxymethylation of amino and imino groups of the nucleic acid bases. This is followed by a slow reaction resulting in methylene bridge formation between bases. Formaldehyde also forms cross-linking of proteins to DNA. This therefore relates to the reduced quality and quantity of the DNA template for PCR. Cross-linking mainly alters proteins in the tertiary and quaternary structure (114).

There are two main types of fixation for histology. These are physical fixation, chemical fixation and immunolabeling fixation. The mechanism for physical fixation generally involves a physical change in the specimen. Common examples of physical fixation are freezing and heating. Chemical fixation involves the use of chemicals which stabilise certain molecules in the specimen. These molecules may include proteins, nucleic acid or mucosubstances which are made insoluble, immobilising them in the specimen and preserving the overall structure. Chemical fixatives can fix biological specimens by physical or chemical action. Physical action implies that stabilization of the specimens' components takes place without a direct chemical reaction between fixative and proteins. Chemical action implies that the fixative stabilizes the biological specimen by direct chemical action of the fixative with the certain components of the specimen (115)

The main chemical fixation types include aldehydes, alcohols, oxidising agents, mercurials and picrates. Aldehyde fixatives include formaldehyde and glutaraldehyde. These fixatives fix by cross-linking with proteins, especially lysine residues (115). Cross-linking involves binding amino groups and forming methylene bridges via small polymers of the fixative (115). Soluble proteins are bound to

structural proteins making them insoluble and stable, preserving overall structure (117, 116). Mercurials contain mercuric chloride and fix tissues via an unknown mechanism. Picrates, which contain picric acid, also have an unknown mechanism of action (115). Oxidising agents include osmium tetroxide which cross-links proteins via oxidation (115).

Alcohol fixatives are water-substituting fixatives and work by precipitating and denaturing proteins in the specimen. A substantial concentration of the fixative must be attained before effective precipitation or coagulation begins, leaving ample time for disruption of cell organization. Thus fixation depends on the accumulation of a sufficient concentration within the tissue to precipitate and denature proteins. Ethanol adequate fixation begins at concentration of at least 50-60%. Methanol at least 80%. Chemical action implies that the fixative stabilizes the biological specimen by direct chemical action of the fixative with the proteins - Commercial formalin substitutes for histopathology. Other denaturing type fixatives, which operate by the same mechanism as the alcohol fixatives include glycol, acetone, acetate and acetic acid (118).

1.5.1.1 Alcohol based fixatives

Alcohol fixatives are water-substituting fixatives and work by precipitating and denaturing proteins in the specimen. A substantial concentration of the fixative must be attained before effective precipitation or coagulation begins, leaving ample time for disruption of cell organization. Thus fixation depends on the accumulation of a sufficient concentration within the tissue to precipitate and denature proteins. Ethanol adequate fixation begins at concentration of at least 50-60%. Methanol at least 80%. Chemical action implies that the fixative stabilizes the biological specimen by direct chemical action of the fixative with the proteins - Commercial formalin substitutes for histopathology. Other denaturing type fixatives, which operate by the same mechanism as the alcohol fixatives include glycol, acetone, acetate and acetic acid (118).

Alcohol based fixatives have been used in histology for many years, particularly for cytological smears. A cytological smear is a thin tissue or blood sample which is smeared or spread usually on a glass slide. The advantage of alcohol based fixatives over other fixatives is that they are fast acting. However, they are also more damaging to tissues and are not able to be used on larger tissues (2).

For forensic purposes, tissue damage is a non issue, as long as the fixative does not destroy haemoglobin, DNA or RNA. Also, because blood stains are very thin, alcohol based fixatives will not have the penetration issues which surround the fixing of biological tissue (2).

The alcohol fixative utilised in this thesis was a combination of methanol and acetone at a ratio of 30:70. This combination was found experimentally to be the best at preventing distortion to blood patterns. Combinations of ethanol, methanol and acetone at varying ratios were attempted (120).

1.5.1.2 Zinc based fixatives

Zinc compounds are superior over other compounds used for fixation because they are non-toxic, inexpensive, non-carcinogenic, not temperature sensitive and have improved DNA, RNA and protein yield compared to formaldehyde (121).
Zinc based fixatives are nonhazardous and therefore require no special arrangements for storage. They are easy to prepare and relatively inexpensive. DNA and RNA can also be recovered from samples fixed with zinc based fixatives (114).

Several studies have been conducted evaluating the use of ZBF in comparison to other fixatives. D. Lykidis 2007 evaluated several zinc fixatives based on the original ZBF Z2 which is 0.5% zinc chloride, 0.5% zinc acetate, 0.05% calcium acetate in 0.1M Tris-HCl pH 6.4-6.7. Modifications of the Z2 formula were where zinc acetate was replaced by zinc trifluoroacetate in Z7, zinc citrate in Z8, zinc trifluoroacetate DMSO in Z16, zinc tartrate in z17, zinc tartrate DMSO in z18 and zinc isovalerate in z19. Overall it was found that Z7 (zinc trifluoroacetate addition) was the best at fixing

tussues. The authors also investigated the use of other metal ions as replacement for zinc but all the metal ions investigated were found not to be as good (117).

A major disadvantage of zinc based fixatives is that the buffer commonly used is tris-hydrochloric acid. HCl, being an acid, could quench the chemoluminescence of luminol and the fluorescence of fluorescein thus reducing their sensitivity.

In this thesis, two zinc formulas were chosen HCl was excluded from the zinc formula to limit the quenching of chemiluminescence. These two formulas were named Z1 and Z2. The Z1 fixative contained zinc acetate, zinc chloride and calcium acetate. Z2 contained zinc trifluoroacetate, zinc chloride and calcium acetate.

Zinc acetate relies on denaturing proteins and nucleic acids through changes in pH or via salt formation. Zinc chloride- non-coagulant crosslinking fixative- crosslinks within and between proteins and nucleic acids (119).

1.5.2 Retention Aids- Xanthan Gum

Xanthan gum is derived from the polysaccharide of the organism *Xanthomonas campestris.* Xanthan gum was discovered in the 1950's and during the 1960's was researched extensively due to its ability to supplement other known natural and synthetic water-soluble gums (121). The function of the polysaccharide secreted by Xanthomonas camprestris is not well understood but is thought to either act as a protective envelope to protect from dehydration and UV, facilitate bacterial colonization of plant cell surfaces or destroy protective structure of target plants to facilitate bacterial infection spread (122)

1.5.2.1 Properties

Xanthan gum is highly soluble in both hot and cold water. When made into solution, the solutions are highly viscous even at low polymer concentrations. Xanthan gum

solutions are also non-Newtonian and exhibit pseudoplastic behaviour (also called shear thinning). This means that as shear stress and shear rate increases, the viscosity decreases (121). Viscosity is a measure of flow resistance in a liquid. The higher the viscosity, the more resistant the fluid is to flow. A shear stress is a force applied parallel or tangentially to the face of a material as opposed to perpendicularly applied stress. A shear rate is the rate at which a shear stress is applied. In the case of xanthan, shear stress is the force which makes part of the xanthan solution slide past the other part. Therefore xanthan gum solution can be poured because the viscosity will decrease and then once the shear force is removed, the viscosity increases again. The viscosity also depends on temperature, biopolymer concentration, concentration of salts and pH. On the whole, viscosity decreases with increasing temperature. The viscosity of xanthan solutions increases strongly with increasing concentration of xanthan. Xantham solution viscosity is unaffected by pH changes between pH1 and 13 (121).

1.5.2.2 Health and safety

Xanthan gum is non-toxic, does not inhibit growth, is non-sensitizing, does not cause skin or eye irritation and is not degraded in the digestive system (121,122). Xanthan is used in a wide variety of foods including salad dressings, syrups, dairy products, frozen goods and baked goods due to reasons such as emulsion and suspension stabilization, temperature stability, compatibility with food ingredients and most metal salts (121,122), thickening aqueous solutions and pseudoplastic rheological properties. These properties are also beneficial for use in other industry including pharmaceuticals, cosmetics, agriculture, textile, ceramic glazes, slurry explosives, petroleum production and enhanced oil recovery (121).

1.6 Fluorescein

Fluorescein was first synthesized by von Baeyer in 1871. Fluorescein has been extensively utilised in many areas of science including opthamology, tracking

underwater rivers and immunology. The first reported use of fluorescein for the detection of blood was in 1910 when Fleig used fluorescein to detect blood in the urine of patients (95). Cheeseman in 1995 was the first to use fluorescein cover large areas in crime scenes for the detection of blood in the forensic setting (72).

1.6.1 Fluorescein Reaction with Blood

Before fluorescein is applied to area suspected of containing blood, fluorescein is reduced to fluorescin. Fluorescein is reduced in an alkaline solution containing zinc (72). When fluorescein is applied to a bloodstain, it is accompanied by the addition of hydrogen peroxide to the blood stain (8,9). The catalytic activity of heme causes the oxidation of hydrogen peroxide which produces oxidising agent. These oxidising agents enhance the conversion of fluorescin to fluorescein (8,123).

Fluorescein emission can then be viewed using an alternative ultra violet light source between 425-485nm (8,1) and by wearing orange safety goggles. The goggles are needed to filter the light (92). The emission spectrum of fluorescein shows two bands with maxima at 425 and 520nm. The later band, 520nm, is the wavelength specific to fluorescein emission (95).

Fluorescin   Hydrogen   Fluorescein
                    Peroxide
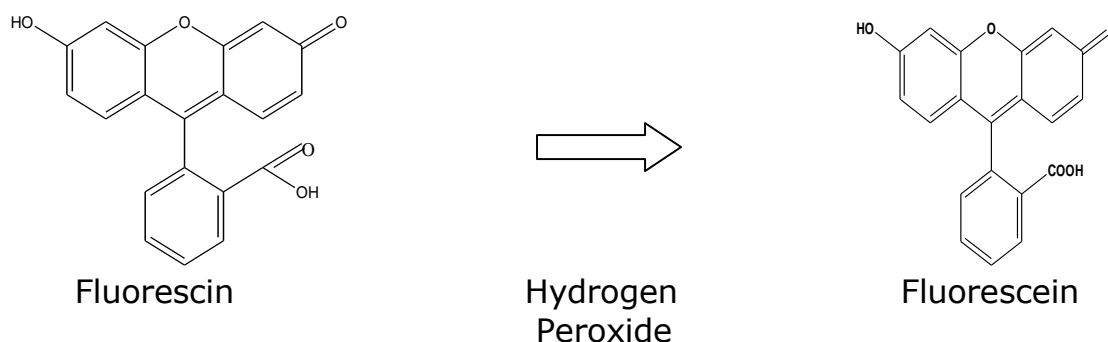
Figure 1.6: Conversion of Fluorescin to Fluorescein

1.6.2 Advantages

One advantage of a fluorescent reaction of a chemiluminescent reaction is that processing of stains with fluorescien can be achieved in a lighted environment. This makes viewing the bloodstain in context with the crime scene easier (126). Another advantage is that the reaction persists longer than luminol. This gives more time for observing and photographing the reaction than with luminol (125). Another advantage is that xanthan gum can be added to fluorescin preparation as a thickener to reduce running and pooling of bloodstains (8,1). One study concluded that it was easier to photograph a fluorescien reaction than luminol with bloodstains which have been exposed to a fire due to the sooty conditions. They claimed that luminol required additional amounts of reagent whereas less reagent was used for fluorescein (129).

There are conflicting studies on the sensitivity of the fluorescein reaction with blood. One study found that on non-absorbent surfaces, stains were detectable at 1:100,000 dilutions and on absorbent surfaces, no more than 1:100, same as with luminol (92). In a study by Cheeseman (1999) it was concluded that fluorescein had an average of approximately four fold increased bloodstain detection sensitivity compared to luminol (127). In another study, a positive result was obtained on diluted bloodstains of up to $1:10^7$ on many different substrata (95)

1.6.3 Effect on DNA

There is no evidence of DNA degradation caused by fluorescein. Budowle et al conducted a study to determine if DNA could still be extracted from fluorescein and luminol treated bloodstains using several STR profiling kits on many substrate types. They concluded that material contaminated with fluorescein or luminol may be successfully typed as long as sufficient quantity and quality DNA is recovered and there was no evidence of DNA degradation (92)

Martin and Cahill (2004) evaluated the recovery of DNA from bloodstains treated by fluorescien on blue denim in the D18S51 loci. They concluded that DNA was successfully extracted from the substrates after fluorescein treatment (129).

Budowle et al 2000 undertook studies on the effect of fluorescein on STR testing and concluded that the results showed no evidence of DNA degradation (72)

1.6.4 Disadvantages

Background staining is prevalent with application of fluorescien. However, a portion of substrate without blood on it can be treated with just the reagent to observe potential background staining prior to applying fluorescien (92).

Another disadvantage is that visualization of fluorescein treated bloodstains requires an alternative light source at 450nm and requires the use of orange safety goggles (8,127). Luminol on the other hand does not require any other light source to view the reaction. Some common multi-wavelength alternative light sources (polilights) are bulky and require electricity to run. Therefore issues could arise over the portability of ALS's to and around a crime scene. Also if a crime scene is in a remote location, a potable power generator may be needed. However, there are some ALS instruments that come in the form of torches which are not bulky and run on battery. Most of these instruments only emit one wavelength, for fluorescene viewing. Additionally, ALS can be expensive, or in any case, an expense which luminol does not require as well as the orange safety goggles and filters for photography (126)

Fluorescein, like luminol, will react with any substance containing peroxidises such as iron, copper and plant peroxidises in a similar way that it does with blood (101). Unlike luminol, fluorescein does not fluoresce with bleach (72). Background fluorescence can occur with substrates which contain highly fluorescent materials. This decreases the contrast between the reaction and background making the fluorescent reaction harder to observe. Background fluorescent increases over time which poses some implications for photographing a fluorescent reaction (72).

Up until recently one of the main disadvantages of fluorescien was that a field-ready reagent was not commercially available and therefore required laboratory preparation. Therefore use of fluorescien has been limited in its use in the field (92). However, there are now commercially available kits where fluorescien is made in the form of a activation tablet which can be dissolved with water making fluorescien more applicable for field work. However, hydrogen peroxide is also needed to be sprayed onto the bloodstain making bloodstain detection by fluorescein a two step process (92). One such commercially available kit is Hemascein, developed by Abacus Diagnostic which is described in the next chapter.

2. Materials

2.1 Laboratories

All experimental work undertaken in this thesis was conducted at the Institute of Environmental Science & Research Limited (ESR), Mt Albert, Auckland, New Zealand. Specifically, the experiments were confined to laboratory 5 of the Forensic Service Centre laboratories and DNA laboratories in the Forensic Biology department. Laboratory 5 is a windowless room, providing an excellent dark environment to view and photograph the luminol reaction.

2.2 Blood

All experimental work requiring blood was performed using pig blood except for blood required for the DNA and mRNA analysis experiments whereby human blood was used.

2.2.1 Pig Blood

The pig blood utilized for experimental work was obtained from Auckland Meat Processors Limited in Otahuhu, Auckland, New Zealand. The blood was collected straight from the neck wound of a pig by staff at the meat processors plant into a 2L or 1L Schott glass bottle. The bottles, prior to collection, were cleaned in Virkon by an ESR technician and contained 10 grams of EDTA per 1 litre of blood. The EDTA served as an anticoagulant. On retuning to ESR, the bottles of blood were dated, labelled and stored in the Service Centre cold room at a temperature of

approximately 4°C until needed. Blood sample "Pig 1" was obtained on 3 May 2010 and "Pig 2" was obtained on 9 September 2010.

2.2.2 Human Blood

The human blood utilized in experimental work was my own acquired via a small incision to the thigh using a sterile scalpel blade. Prior to making the incision the area was swabbed with alcohol to prevent contamination. 200µl of blood was then collected via a calibrated pipette using sterile pipette tips and transferred to a Trigene cleaned glass dish. This was repeated with 100µl of blood.

The blood was used immediately for experimental setup so therefore no anticoagulant was used. Gloves were used at all time to prevent both contamination from any contaminants which may have been on my hands and DNA and mRNA which may be transferred by touch. This is important because the experiments were designed to only evaluate DNA and mRNA which originated from the blood. As a precaution, after making the incision and transferring the blood to the glass dish, the gloves were changed in case blood was transferred onto the gloves during the blood attaining process. Fresh gloves were put on and experimental set up continued.

2.3 Substrates

2.3.1 Fabric

White single sheets were purchased from The Warehouse in Balmoral, Auckland. The Manufacturer of the sheets is Red Stamp based in Australia. The sheets consisted of 52% cotton and 48% polyester. To limit contamination, the white sheets were taken into the lab without opening the manufactures wrapping. Once in the lab, the sheets were cut into approximately 5x5 cm squares using scissors cleaned with 70% ethanol followed by Virkon. The squares that were not used immediately for experimental work were sealed in a plastic sheath and stored in the office area of the Service Centre.

Cotton/polyester fabric was chosen for some of the experiments due to the porous nature of this substrate and because it is commonly found in crime scenes. Bloodstains on this type of substrate retain their shape when sprayed with the blood detecting reagents used in the experiments, which was desired for some of the experiments.

2.3.2 Tiles

The Tiles were donated by The Tile Depot in Manukau, Auckland. They are glazed ceramic tiles composed of porcelain and a mixture of clays. The tiles were chosen based mainly on availability and quantity needed for experimental work. The tiles were cut by a staff member of The Tile Depot into 5 by 5cm squares. They were then transported to ESR and stored in the Service Centre office area till used in experiments. Prior to experimental work, the tiles were wiped clean with Virkon followed by 70% EtOH to eliminate any contamination.

A glaze is a glass material which melts and adheres to the surface of the tile and is primarily required to provide moisture resistance and decoration. This glazing was an important attribute in parts of my experimental work as it provided an example of smooth, non-porous surfaces of which may be encountered in crime scenes (130).

2.3.3 Vinyl

Vinyl floorcovering sheets were donated by Flooring First in Manukau, Auckland. Similarly to the ceramic tiles, the vinyl sheets were chosen based on availability and the quantity needed for experimental work. The vinyl sheets were then cut into roughly 5 by 5cm squares with a craft knife cleaned with 70% ethanol and stored in the Service Centre office area until needed for experimental work. Prior to experimental work, the vinyl squares were wiped down with Virkon followed by 75% EtOH to eliminate contamination.

The vinyl flooring provided another example of a smooth, non-porous surface. Also, vinyl is one of the most common flooring surfaces and as such would be commonly encountered at a crime scene.

2.3.4 Graph Paper

The graph paper utilized in the experiments was purchased from The Warehouse Stationary in Auckland CBD. The graph paper contains major grid lines intersecting to form 10mm^2 squares, intermediate grid lines intersecting to form 5mm^2 squares and minor grid lines intersecting to form 1mm^2 squares.

2.4 Blood Detecting Reagents

2.4.1 Grodsky luminol

Grodsky's luminol was utilized in experimental work as it is the luminol commonly used at ESR and was prepared in accordance with ESR protocol (112).

Table 2.1 shows the amount of each constituent of the Grodsky luminol formula

Constituent	Amount	Company	Batch No.	Country
Sodium perborate	1.75g	Adrich	03630DJ	Germany
Sodium Carbonate	12.5g	Merick	1.06392.0500	Germany
Luminol	0.25g	Sigma	MKAA3480	Germany
Millipore ELIX10 water	250ml	ESR		New Zealand

Table 2.1: Grodsky luminol formula as used in experimental work

Two separate solutions were prepared initially and then combined to form the working reagent. Solution A contained 1.75g sodium perborate dissolved in 150ml of sterile water. Solution B contained 12.5g sodium carbonate and 0.25g luminol dissolved in 150ml of sterile water. Each solution was mixed well by shaking before

being mixed together in equal volumes prior to application. Luminol was made up fresh for each experiment and used immediately.

2.4.2 Bluestar Magnum

Bluestar Magnum is a luminol based formula and was obtained from Bluestar®, Monte-Carlo, Monaco and prepared as instructed by the manufacturer. One third of the Bluestar forensic magnum chemiluminescent solution was poured into a spray bottle. One of the three activation tablets (oxidizing tablets) was added and dissolved by gentle stirring. Because Bluestar Magnum consists of three activation tablets, one activation tablet can be used with a third of the chemiluminescent solution at a time. Bluestar was made up fresh for each experiment and used immediately.

2.4.3 Hemascein

Hemascein is a flourescein based formula and was obtained from Abacus Diagnostics®, West Hills, USA and prepared as instructed by the manufacturer. 5ml of distilled water was added to the hemascein powder vial and was mixed. This was called the hemacein stock solution. 1ml of this solution was added to 100 ml of distilled water and mixed in a sprayer. This was the hemacein working solution. 30% Hydrogen peroxide was obtained from BioLab, Australia. The Hydrogen peroxide was diluted to a concentration of 3% and placed in a separate sprayer. Because there was 5ml of the stock solution, 100ml of the hemacein working solution could be made five times. Hemascein was made up fresh for each experiment and used immediately.

2.4.4 Lumiscene

Lumiscene is a fluorescein and luminol based formula and was obtained from Loci Forensic Products, Nieuw-Vennep, Netherlands and prepared as instructed by the manufacturer. One of the two activation tablets were added to one half of the water based lumiscene stock solution and was shaken gently for 1 minute at 5, 10 and 15 minute intervals to activate the lumiscene. The lumiscene was then poured in a spray bottle. Because lumiscene consists of two activation tablets, one activation

tablet can be used with half of the stock solution at a time. Lumiscene was made up fresh for each experiment and used immediately.

2.4.5 Lumiscene Ultra

Lumiscene Ultra is also a fluorescein and luminol based formula obtained from Loci Forensic Products and was prepared as instructed by the manufacture. The preparation of Lumiscene Ultra was the same as for lumiscene except there was only one activation tablet. Lumiscene was made up fresh for each experiment and used immediately

2.5 Spraying apparatus

2.5.1 Pump sprayer

The pump sprayer was acquired from the Service Centre Laboratory at ESR as this sprayer is most commonly used at ESR for spraying luminol. The pump sprayer works on a compression based system whereby air in a bottle which contains the spray solution is pressurised using a built in hand pump. When the trigger is depressed, the compressed air forces the solution up through the lance and out the nozzle. As the liquid is sprayed, the air expands to fill the vacated space and the pressure lowers which consequently reduces the pressure and volume of liquid sprayed. Therefore the pressure utilized in these types of sprayers is not constant over time (131).

2.5.2 ABA spray

The ABA spray was obtained from Abacus Diagnostics®, West Hills, USA. The ABA spray is a compression sprayer and operates similarly to the pump sprayer but on a much smaller scale.

2.5.3 ABA finger spray

The ABA finger spray was obtained from Abacus Diagnostics®, West Hills, USA. The finger spryer operates similarly to the sprayer of a perfume bottle. As the nozzle is depressed, the pressure inside the reservoir changes which causes the liquid in the reservoir to be pulled up quickly through the lance. The liquid is then forced through a screen in the nozzle which contains small outlets, dispersing the liquid in a fine spray.

2.5.4 ECO spray

The ECO spray was obtained from Bluestar®, Monte-Carlo, Monaco. The ECO spray consists of a pressure reservoir and a reagent reservoir. When the top button is depressed, reagent is sucked up from the reservoir through the lance as simultaneously a gas propellant is released from the pressure reservoir. The gas nebulises the reagent in a vapour phase with a pressure of 64 psi (4.5 kg/cm2) as the reagent emerges from the nozzle. The gas propellant used in the ECO spray is 1,1,1,2-tetrafluoroethane (132)

2.5.5 Spray gun

The air gun utilized in the experiments was a gravity feed detailing spray gun model w7013 (Hindin Marquip Ltd) connected to a compressed tank of nitrogen gas (BOC Gases New Zealand Limited). This Spray gun is designed to force a 10-25psi (0.7-1.76 Kg/cm2) airflow in through the vase of the gun via a regulator attached to a standard gas tank. The gun trigger when half depressed releases the air from the base past the liquid chamber valve to exit through the nozzle. When the trigger is fully depressed, the valve to the liquid holding chamber is opened and the luminol solution will flow under gravity feed into the airstream creating a fine aerosol spray. This spraygun has three built in regulators which control the spray width, airflow speed and solution flow speed. This gives precise control over the amount of reagent that is delivered to the target. (74)

2.5.5.1 Spray gun Calibration

A calibration was undertaken to evaluate whether the spray gun sprays a constant volume of liquid in a given time period. This is important for experiments utilising the spray gun as the amount of luminol or fluorescein applied to a bloodstain is a determining factor in the outcome of the reaction. To eliminate the influence of this factor, the spray gun was utilised for its precise control over how much liquid is dispensed.

Firstly, a solution containing phenolphthalein and sodium carbonate was used to visualise the spray mist. The desired spray mist was achieved via alteration of the three inbuilt regulators. The remaining phenolphthalein solution was poured out of the reservoir and replaced by 60ml of phenolphthalein solution. The trigger of the spray gun was fully depressed and the phenolphthalein solution was sprayed continuously for 30 seconds into a beaker. The volume of phenolphthalein solution in the beaker was measured and recorded. The reservoir was then emptied and the measurement repeated 10 times to produce a volume average and standard deviation.

The results of the calibration are as follows:

Average amount of luminol sprayed after 30s of spraying= 5.22ml

Standard Deviation= 0.179

Anova singe factor test was then undertaken to evaluate the differences between each of the values with respect to time. The results are shown in the table below.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.14422	9	0.016024	0.001793	1	3.020383
Within Groups	89.3551	10	8.93551			
Total	89.49932	19				

Table 2.2: Anova single factor test of the spray gun calibration

With a p-value of 1, the spray gun is an appropriate application method the null hypothesis that the replicates were statistically different was rejected. Therefore, the spray gun is an appropriate application method for the constant delivery of luminol and fluorescein.

Additionally, the spray gun operates via a gravity feed mechanism therefore the initial volume in the cup may affect the rate of delivery. However the work of E. Kent in the validation of utilising this spray gun for luminol delivery at ESR dispelled this query and found that the initial volume in the cup does not affect the rate of delivery.

2.6 Chemicals

The chemicals used in the experiments apply to those used in the fixative/sheer thinning agent experiments, excluding those chemicals used for making the blood detecting reagents. Table 2.6 outlines all the chemicals used along with supplier information and which fixative/sheer thinning agent they were used in.

	Fixative	Manufacture	Country
Zinc acetate dehydrate	Zinc fixative 1	Sigma-Aldrich	Germany
Zinc trifluoroacetate hydrate	Zinc fixative 2	Aldrich	Germany
Calcium acetate	Zinc fixative 1 and 2	Sigma-Aldrich	Germany
Methanol 100%	Alcohol fixative	Merick	Germany
Acetone	Alcohol fixative	Merick	Germany
Xanthan gum	Sheer thinning 1	Sigma-Aldrich	Germany
ABA fix	Sheer thinning 2	ABA Diagnostics	America

Table 2.3: Chemicals utilised in this study

2.7 DNA Analysis

2.7.1 Extraction

DNA was isolated using the DNA IQ™ system from Promega. This system includes a lysis buffer, wash buffer, elution buffer and a paramagnetic resin to purify DNA (133).

2.7.2 Quantitation

The amount of DNA acquired from each sample was quantified using Quantifiler™ Real Time PCR quantification kit from Applied Biosystems, Foster City, CA. This system includes the Quantifiler® reaction mix, Qantifiler® primer mix and DNA standards.

2.7.3 STR Amplification

STR Amplification was achieved using the AMPF/STR)® Identifiler® PCR Amplificaiton Kit from Applied Biosystems, Foster City, CA. This system includes the AmpF/STR® PCR reaction mix, AmpF/STR® Identifiler™ Primer Set, AmpliTaq Gold DNA Polymerase, AmpF/STR® Control DNA and AmpF/STR® Identifiler™ Allelic Ladder. The AmpF/STR® Identifiler™ Amplification kit amplifies 15 tetranucleotide repeat loci and the Amelogenin gender determining marker in a single STR multiplex assay amplification (134).

2.8 Cameras and Camera Software

All photographs were taken with a Nikon D100 camera with an ASF-S Zoom Nikkor ED24-85mm f/3.5-4.5 G-type IF lens. Camera settings for the various experiments are discussed in more detail in the next chapter pertaining to methods.

Images were edited either using Nikon Editor 6, Picasa 3, Photoshop CS4 (64 Bit) or Matlab 2010A, depending on what was required for analysis.

2.9 Measurements

All measurements were taken in the Service Centre preparation room or in laboratory 5 at ESR using calibrated equipment except for the filtered sterile water which was measured via measuring cylinders.

2.9.1 Scales

All mass measurements for weighing out the chemicals used in experimental work were taken using a Mettler PC4400 electronic scale. This scale has an accuracy of ±0.01g and undergoes monthly tolerance checks. Known weights are checked within the allowed ±0.05g tolerance limit from the expected weight. This machine also had a six monthly repeatability check conducted on 2/11/10, a yearly service check on 9/11/10 and a 3 year full calibration on 10/11/09.

2.9.2 Pipettes

All volume measurements for blood were taken via a calibrated Eppendorf Research pipette which had a 6 monthly repeatability check on 26/8/10.

2.9.3 Time

All time measurements were taken by a calibrated Quantum timer which was last calibrated in may 2010 as part of a yearly repeatability check.

2.9.4 Blood blocks

To assist in the semi-quantitative measurements in the morphoanalytical studies, blocks with a series of lines were made and self-termed 'blood blocks'. Three blood blocks were made and are depicted in figure 2.1.

Figure 2.1: Blood blocks. From the left, 3mm, 2mm and 1mm

As shown in the picture, one of the blocks consists of 3mm lines with 3mm troughs; another consists of 2mm lines with 2mm troughs and the last, 1mm lines with 1mm troughs. Measurements were made via a ruler and square to make parallel lines of the desired width. The lines and troughs were then created primarily using a hack saw and a fret saw. More intricate lines such as the 1mm lines were created using a fret saw and sand paper.

2.9.4.1 Blood Block Calibration

An attempt to calibrate the blood blocks was undertaken to assess their reproducibility. A 1:1000 blood dilution was made up and poured into a Virkon cleaned, wide rimmed and shallow glass dish. The 3mm blood block was dipped into the solution so that approximately one 2.5 cm of the block was submerged in liquid. The block was then immediately dabbed once onto absorbent tissue paper and transferred to a tile. This was repeated in duplicate for all the block except for the 2mm block was dabbed twice onto absorbent tissue paper and the 1mm block which was dabbed three times. The amount of dabbing for each block was predetermined experimentally to achieve the best resolution between the blood lines for each block as the blood spreads to a certain extent. A dilution of 1:1000 was used because at higher dilutions the luminol only reacts with the edge of the lines.

At lower dilutions, the blood tends to spread more which would interfere with the resolution of the lines.

The samples were left to dry overnight. Once dry, the samples were photographed with right angled rulers surrounding the sample. The images were processed in Photoshop and the Photoshop ruler was calibrated to the increments on the right angled rulers. The width of each line and trough was then measured based on the Photoshop ruler increments set to measure millimetres. The lines and troughs of different images of samples stamped with the same block were all measured in the same places.

For the 3mm block, the total height of each image was divided into 4 sections of equal size by axis lines running perpendicular to the blood lines. The measurements were taken where the three innermost axis lines intersected the blood lines and along each axis line to measure the width of the blood lines and troughs. For the 2mm and 1mm blocks, the images were divided into three sections of equal size and the two innermost axis lines were utilised to ensure measurements were taken from the same places in each image. The measurements at each intersecting axis line for each line and trough in each image and for each block were recorded in an excel spreadsheet.

The results of this calibration are portrayed in the following table:

	Average (mm)	Standard Deviation	Line numbers
3mm Lines	3.69	0.420	4-10
3mm Spaces	2.16	0.349	4-5—8-9
2mm Lines	2.86	0.360	1-8
2mm Spaces	1.03	0.409	1-2—7-8
1mm Lines	1.74	0.176	1-10
1mm Spaces	0.467	0.131	1-2—9-10

Table 2.4 Showing the results of the blood block calibration. For the spaces, the line numbers relate firstly to the space between the first two visible lines through to the space between the last two visible lines.

Notably, the averages for the 3mm, 2mm and 1mm lines are higher than 3mm, 2mm and 1mm, respectively. Similarly, the averages for the 3mm, 2mm and 1mm spaces are lower than 3mm, 2mm and 1mm. The reason for this is that when the blood is transferred to the non-porous surface, a certain amount of spreading is observed which thickens the lines and narrows the spaces between the lines. This would not have a profound affect on results as the lines can still be utilised for comparative purposes. Variation between the samples of the same type was observed as shown by the standard deviation. However, this variation is reasonable as Photoshop measures lines to 1 tenth of a millimetre and the measurements used to create the blocks had increments of 1mm with a possibility of viewing half a mm by sight. Therefore, an allowance of 0.5mm either way of the measured value was acknowledged for all the experiments utilising the blood blocks.

Also what is apparent from the results of this calibration. is that some of the lines consistently failed to produce viable lines for measurement. These lines were omitted from any experiment involving the measurement of these lines. As shown in table 2.4, the omitted lines include lines 1-3 on the 3mm block (figure 2.2) and 9-10 on the 2mm block (figure 2.3).

Figure 2.2: 3mm Blood Block

Figure 2.3: 2mm Blood Block

Figure 2.4: 1mm Blood Block

3. Methods

3.1 Health and Safety

The ESR Health and Safety protocols require all persons present in the Service Centre laboratory to wear safety glasses, a mask, a gown, covered-toe shoes and gloves. As such, this precautionary personal safety wear was deemed sufficient during the preparation and application of the various reagents when performing the experiments.

Additionally, orange safety goggles were worn when applying hemascein to the samples due to potential damage to unprotected eyes when using the UV spectra of the polilight®. The polilight® is needed to visualise the fluorescent reaction of hemascein with blood.

3.2 Sensitivity Experiment

Sensitivity is an important aspect when developing new latent blood detecting formulas as blood at crime scenes can be quite diluted due to attempts of "cleaning up". Detecting blood at extremely low dilutions requires the blood detecting reagent to react with very small amounts of haem present. In this experiment the sensitivity of each reagent was examined. The reagents in this experiment include Grodsky's luminol, Bluestar Magnum, Lumiscene, Lumiscene ULTRA and Hemascein. Descriptions of these reagents can be found in section 2.4.

Aim: To assess the relative sensitivity both quantitatively and qualitatively of certain reagents and to define a limit of detection.

A dilution series using pig blood and distilled sterile water was made with final blood concentrations of 1:1000, 1:5000, 1:10,000, 8:100,000, 6:100,000. 4:100,000, 2:100,000, 1:100,000, 1:500,000 and 1:1,000,000. Measured by a calibrated pasture pipette, 100µl of each concentration was used to stain the centre of 5x5 cm squares of cotton/polyester fabric. The luminol reaction is dependant on the amount of blood, with a more intense chemiluminescent reaction produced by a higher concentration of haemoglobin. 100µl was chosen because it proved an adequate amount to cause a chemiluminescent reaction with luminol without the light produced saturating the camera sensors at higher concentrations of blood. Care was taken to deposit the dilute blood onto the cotton squares in an attempt to keep the shape of the stains uniform. For each reagent, excluding Hemascein, three such stains were made for each dilution making a total of 120 samples. For the Hemascein samples, due to complications, two samples were made for each dilution making an additional 20 samples.

Positive and negative controls were also created. The positive control consisted of a cotton/polyester square with a 1:100 blood stain in its centre and the negative control consisted of one blank cotton/polyester square. One positive control and one negative control were used for each reagent. All samples, as well as the controls, were prepared in the afternoon and left overnight to dry to be treated with each of the reagents the following day.

Each of the samples were set up as shown in Figure 3.1

Figure 3.1: Setup for Sensitivity experiments

As shown, the bloodstained cloth, calibrated spray gun and camera were set up in fixed positions. The cloth was held in place by a bulldog clip ensuring that the blood stain was in the centre of the camera view and did not move during spraying. The camera settings were set in accordance with ESR protocol for luminol photography and experimentally found to achieve the best result for fluorescence photography. These parameters are shown in table 3.1

Camera setting	Luminol Photography	Fluorescence Photography

Exposure time	30 sec	10 sec
Aperture	f / 4.5	f / 9
White balance	Flash mode	Flash mode
ISO equivalent	800	200
Focal length	50mm	50mm
Format	RAW (.nef)	RAW (.nef)

Table 3.1: Camera settings chemiluminescence and fluorescent reactions.

The camera was set at a distance of 62cm from the surface of the cloth. The spray gun was calibrated as shown in section 2.5.5.1 and was set at a fixed angle with the nozzle directed at the centre of the cloth at a set distance away from the cloth. This was important as the luminol and fluorescein reaction is affected by the amount of reagent applied to a bloodstain. Using a calibrated spray gun at a fixed position minimised this variability.

The lights in the lab room were then turned off. Each reagent was sprayed onto the bloodstain for three seconds, timed with a calibrated timer. The camera was engaged immediately after the 3 second spray via a cable shutter release attached to the camera. The cable shutter release was used to minimise possible movement to the camera which could be caused by pressing the camera button to capture the image. The lights remained off until the exposure concluded 30 seconds later. The cloth was then removed and replaced by the next cloth and the process repeated until all samples were sprayed and photographed.

For the Hemascein samples, the polilight was used at a wavelength of 415nm and at a fixed distance from the sample with the main stream of light from the polilight near the sample but not directly focused onto the sample. This was done so as to not saturate the sample with light. A field blank image was taken by the camera which showed the area of illumination by the polilight without a sample present. The camera settings for the Hemascein experiments were set as described above for fluorescence photography. These settings were found experimentally to be the best for capturing the Hemascein reaction in this setup.

The lights in the lab, except for the polilight were then turned off. After applying the reagent for 3 seconds the samples were left for 1 minute for the fluorescence to develop in accordance with (92). A photograph was then taken via cable shutter release and lights remained off until the exposure concluded 10 seconds later. The cotton cloth was then removed and replaced by the next cloth and the process repeated until all sampled were sprayed and photographed. Figure 3.2 shows the experimental setup for the Hemascein treated samples.

Figure 3.2: Experimental setup for the Hemascein treated samples

Additionally a field blank image was captured. This involved photographing a blank piece of black paper with the polilight on and in the exact same position as it was for the experiments. The filter was taken off the camera and an image taken with 1/2s exposure time, f/9.0 and ISO 200 camera settings. The field blank was taken to show the distribution of light from the polilight over the experimental area and was important for the subsequent processing of the hemascein images in Matlab.

Basic image processing was achieved in Photoshop CS4, which involved adjusting the contrast and colour to obtain an enhanced image. The photographs were then processed in Matlab 2010A to quantitatively evaluate the intensity of the light captured during the reaction and to create qualitatively comparable images. Matlab code for image processing in this thesis was written by Dr Gordon Miskely of the Chemistry department in The University of Auckland.

3.2.1 Matlab Processing

The RAW images were converted to tiff files for in Photoshop for processing in Matlab. Templates were then created to extract the blue and green pixels from a Bayer pattern. The region of interest, where the reaction occurs, was defined by a coordinated system of pixels. The blue pixel image is half the linear size of the original image and the green pixel image is the same size due to the representation of blue and green pixels in the camera sensor. Therefore the selected region for the green pixels was twice the size of the blue. An initial 3x3 median filter was applied to

the whole image as a way of reducing noise interference and smoothing the image. A set of images were then saved as "un-averaged". A 15x15 area was then selected in the middle of the image as the middle of the image harbours the pixels pertaining to the reaction, due to experimental setup. Once the area of selection has been defined the green pixels were then selected. Missing green values are calculated using the median of the four neighbouring pixels. This is followed by a 3x3 median filter. The blue pixels were then selected. Geometric averaging was then performed to give a mean value of intensity over all the pixels in the selected 15x15 area. Another set of images were then saved as "averaged". Geometric averaging was favoured over arithmetic averaging because geometric averaging is more suited to data which is not normally distributed. The 15x15 area contains pixels of vast varying intensities with potential to have skewed data. Therefore Geometric averaging was undertaken.

After processing the region of interest, part of the background was selected. Thresholds were then created to enable the noise within the image to be subtracted from the image of the reaction. Two thresholds were created for this purpose. The first threshold, thresholdflag 0, was defined as ((imgBspotmean-2x imgBspotstd) + (imgbkdmean+2x imgBbkgstd))/2; where imgBspotmean is the mean intensity of the selected area of the image, imgBspotstd is the standard deviation of the image intensity, imgbdkmean is the mean intensity of the selected area for the background and imgBbkgstd is the standard deviation of the background intensity for the selected area. However, if imgBspotmean- 2x imgBspotstd was less than imgBbkgmean + 2x imgBbkgstd then a second threshold imgBbkgmean +3x imgBbkgstd was utilised, threshold flag 1. The B in these formulas refers to the blue pixels. When the green pixels were analysed the B was replaced by G. Code was then used to identify the pixels above the thresholds and calculated the means, standard deviations and sizes of the image.

The Hemascein images were processed slightly differently. Initially, the field blank was geometrically averaged using and area of 151x151 to purposely blur the blank. The purpose of this was to reduce detail as only the distribution of light rather than a measure of intensity was needed. The blank was then divided by the intensity of the

pixel with the highest intensity in both directions to yield a ratio image of the blank with values from 0 to 1. Therefore, the brightest pixel in the image would be assigned the value 1 and the dimmest pixel would be assigned the value 0. The images of the reaction are then divided by this ratio image in an attempt to reduce the variation in light intensity from the polilight over the experimental area. The Hemascein images were then processed as the other images through Matlab, first obtaining "un-averaged" images and then 15x15 geometrically averaged images. However, because the white cloth false positively fluoresced to a certain extent, the background selected needed for finding the threshold was part of the cloth known to not contain any blood rather, than the background which the cloth was fastened to.

For all of the reagents, the final intensity value obtained was therefore the geometrically averaged value minus the threshold value. This was a value assigned by Matlab in arbitrary units for the purpose of directly comparing the values obtained for each of the reagents.

The Grodsky, Bluestar Magnum and Lumiscene samples were all processed on the same day and in that order. For each reagent the positive control was processed, first followed by the negative control, then each dilution in triplicate from 1:1000 to 1:1,000,000. Due to changes in the formula, the Lumiscene ULTRA samples were processed at a later date. The Hemascene samples were also processed at a later date due to substrate difficulties. The white cotton/polyester fabric used with the other samples reagents produced a background fluorescence with the polilight that interfered with the hemascein reaction.

An attempt to use dark fabric as opposed to white fabric to eliminate this background interference was undertaken. However, the dark fabric, although made by the same manufacturer and with the same quantities of polyester and cotton, failed to absorb the blood significantly which lead to very weak reactions with hemascein. Further attempts to utilise the dark fabric for the experiment involved washing the sheets in a washing machine with Pyroneg detergent. However, even after a couple of washes, the porosity of the sheets were only very slightly improved and thus unable to be utilised for the experiment. The white sheets, although not

entirely satisfactory, were therefore used instead and care was taken to ensure the best possible results from this substrate.

3.3 Reaction Length Experiment

Typically, the luminol reaction is short lived, around 30s, which gives the analyst little time to photograph the reaction for documentation. Further spraying can be carried out which can allow more time to photograph a reaction. However, this can further distort and dilute a bloodstain. Therefore, a longer luminol reaction would give the analyst more time to detect and examine any latent blood. Another reason why a longer reaction would be beneficial is to help differentiate between a reaction with blood and a false positive reaction. Haem catalysed reactions are generally longer lived than species which can give false positive reactions such as bleach and metal ions. A longer reaction would help further distinguish a positive reaction from a false positive reaction, particularly if the blood is contaminated with these species. Therefore, increasing the longevity of the reaction between blood detecting reagents and blood would be valuable in the detection of latent blood. In this experiment, the reaction length between blood and each reagent was assessed. The reagents in this experiment include Grodsky's luminol, Bluestar Magnum, Lumiscene, Lumiscene ULTRA and Hemascein. Descriptions of these reagents can be found in section 2.4.

Aim: To assess the relative reaction length both quantitatively and qualitatively and to define a reaction length limit of detection for each of the reagents.

A 1:1000 dilution of pig blood was made with sterile distilled water. 100μ of this was transferred onto the centre 5x5 cm cotton/polyester squares, 3 for each reagent making a total of 15 samples. A 1:1000 dilution was used as this dilution provided a sufficient reaction for the entire reagents tested. A positive control consisting of a cotton/polyester square stained with 1:100 diluted pig blood and a negative control consisting of a blank cotton/polyester square was made for each reagent. These

samples were prepared in the afternoon to be sprayed with the reagents the next day. The samples were set up as with the sensitivity experiment with the samples, calibrated spray gun and camera at the defined fixed positions. The camera settings were also unchanged from the sensitivity experiment.

For the Grodsky, Bluestar Magnum, Lumiscene and Lumiscene ULTRA samples, the samples were sprayed for 3 seconds with each reagent and the first exposure taken immediately via cable shutter release. After the first 30 second exposure another 30 second exposure was started immediately. This was repeated until 5 minutes was reached making a total of 10 photos per sample. Additionally, the last sample of each reagent was left for longer before images were captured after 10 minutes, 15 minutes and 20 minutes had elapsed.

For the hemascein samples, a field blank was first taken as with the sensitivity experiment. The samples were sprayed for 3 seconds, then a 10 second exposure image was captured. Additional 10 second exposures were started every 30 seconds after the beginning of the first exposure giving a total of 10 images. Each sample was left longer after the initial 5 minutes with images captured at 10, 15 and 20 seconds.

Also, because fluorescene is known to react for much longer than luminol, one sample was left for 2 hours with 10 second exposures started at 30 minutes, 45 minutes, 1 hour and 2 hours after the initial spray. The sample was left under the polilight with the polilight turned on during all time intervals. Ten second exposures were taken rather than 30 second exposure as in the rest of the reagents because Hemascein is a fluorescein based reagent and requires an external light source to view the reaction. Longer exposures would saturate the camera sensor leading to over exposed images due to the light received by the camera sensor from the external light source. This extended time was not carried out with the luminol based reagents because it was experimentally found that after 30 minutes after the reaction, no emitted light was seen.

The photographs were then processed in Matlab as done with the previous experiment to quantitatively evaluate the intensity of the light emitted during the reaction and to create qualitatively comparable images.

3.4 DNA Analysis

Usually samples are collected for DNA analysis before any reagent is applied to a bloodstain (Dion). However, in some situations, this may not always be possible so it is then important to determine if a particular reagent interferes with the subsequent DNA profiling of bloodstains.

Aim: To obtain DNA profiles from blood stains treated by each of the reagents and to evaluate the extent of DNA degradation, if any, based on results of STR peak height and quantitation results. The reagents in this experiment include Grodsky's luminol, Bluestar Magnum, Lumiscene, Lumiscene ULTRA and Hemascein. Descriptions of these reagents can be found in section 2.4.

DNA extraction, quantification and STR amplification was undertaken by Rachel Fleming (post doctorate research scientist) with assistance from Marita Fallow and Janet Stacey (senior technicians) of the Forensic Biology department at ESR.

3.4.1 Sample Preparation

Samples were made with human blood obtained as described in section To the dish containing 200µl of blood as described at the end of section 2.2.2, 2ml of sterilised, deionised water was quickly added to make a dilution of 1:10. 100µl of this dilution was then transferred to the centre of a cotton square. Twelve samples using this dilution were made, two for each of the reagents and two for water samples. In the other dish containing 100µl of blood, 10ml of filtered deionised water was added making a dilution of 1:100. 100µl of this dilution was then transferred to the centre of a cotton/polyester square. 18 samples using this dilution were made, three for each of the reagents and two for water samples. Each sample was then fixed in position

by a bulldog clip in line with the spray gun nozzle, following the procedure used in the sensitivity and longevity experiments. As with the aforementioned experiments, the samples were sprayed with an assigned reagent for 3 seconds. The hemascein samples were sprayed with hemascein using the spray gun for 3 seconds followed by a two second spray with hydrogen peroxide from the ABA spray. Water samples were made utilising the spray gun to spray the samples for 3 seconds with sterile, deionised water.

The samples were then left to dry overnight and packaged into separate paper envelopes depending on their dilution and reagent type the following morning. The envelopes containing the samples were then collected by Rachel Flemming and taken to the biology department at ESR.

3.4.2 DNA Extraction

DNA was extracted from cut portions of the cotton samples using the DNA IQ™ system from Promega according to the method outlined in the DNA IQ™ System for Casework Manual Protocol (Appendix). This method has been adopted by ESR for forensic DNA casework. This method has several advantages over conventional extraction methods such as organic extraction, Chelex® and silica matrices. DNA IQ™

DNA IQ™ System- Small Sample Casework Protocol- Technical Bulletin (2009) Promega, USA

3.4.3 DNA Quantification

The Quantifiler™ Real Time PCR Quantification kit was utilised to quantify the amount of DNA obtained from the samples and the quantification was monitored using the ABI Prism™ 7500 sequence detection system and SDS software v1.2. DNA quantification using this system was undertaken according to protocol set by the manufacturer and ESR (Appendix). For the 1:10 diluted samples, DNA quantification for the two samples for each reagent was repeated in quadruplicate. For the 1:100 samples, DNA quantification was duplicated.

3.4.4 STR Amplification

The AMPF/STR®Identifiler® PCR Amplification Kit was utilised to analyse the DNA. The method for STR amplification was followed as according to the manufactures recommendations and ESR protocol. The samples were run in the PCR Thermal Cycler 9700 for 28 cycles. The amplified DNA was analysed using the ABI Prism™ 330 DNA analyser according to the manufacturer's recommendations. Analysis of the STR profile was analysed using GeneScan™ analysis software version 3.1 (ABI systems) and the profiles were assigned using Genotyper™ Version 3.7 (ABI systems). (See appendix for STR Amplification protocols). All the samples from the quantification were amplified to yield DNA profiles.

3.5 Spray droplet density and size

There are many different types of sprayers which can be utilised for the application of luminol to forensic case work. The amount of luminol delivered to a blood stain determines how bright the emitted light is. However, if too much luminol is delivered to a stain then the "running" and "pooling" effect or otherwise destruction of blood stain patterns is observed. Therefore, an ideal sprayer for luminol would be a sprayer which can very finely disperse the luminol over an area, keeping the droplet size small so as to avoid running and pooling. Also, the sprayer should be able to deliver the luminol evenly and cover as much of the sample as possible to emit chemiluminescent light showing exactly where the blood stain is. This next section sort to discover which sprayers out of the ones presented in this thesis are most operationally efficient in terms of spray droplet density over a given area and spray droplet size. The sprayers under assessment were a common hand held compression sprayer, the ABA spray, the ABA finger spray, the ECO spray and a nitrogen powered spray gun. Descriptions of these can be found in section 2.5.

Aim: To quantitatively assess the spray droplet density and size of sprayers used for the application of luminol and fluorescein based blood detecting reagents.

A sheet of graph paper with major grid lines intersecting to form 10mm^2 squares, intermediate grid lines intersecting to form 5mm^2 squares and minor grid lines intersecting to form 1mm^2 squares was fastened by a bulldog clip on a vertical stand.

The camera for each sample was in a fixed position. The camera settings are shown in table 3.2 bellow.

Camera setting	Unit
Exposure time	1/80
Aperture	f / 4.5
White balance	Fluorescent
ISO equivalent	800
Focal length	50mm

Table 3.2: Camera settings for spray droplet/density experiments

Three sheets of graph paper were used for each sprayer. The distance between the graph paper and the sprayer was not predetermined and was judged based on the area each sprayer is capable of covering. The hand pump sprayer was sprayed at the greatest distance away from the graph paper because the area which can be covered by the spray mist is the largest. The Eco spray, ABA spray and spray gun were sprayed at similar distances while the ABA finger sprayer was used at the closest distance. Each sprayer was filled with phenolphthalein solution for the visualisation of the spray on the graph paper. Spraying was undertaken as uniformly as possible to cover the sheet of grid paper with phenolphthalein solution. Therefore there were no time restraints on how long the phenolphthalein could be sprayed for. If spraying resulted in "running" of the phenolphthalein solution down the graph paper then a fresh sheet was re-sprayed until 3 sheets per sprayer type were

obtained. As there were three sheets sprayed for each reagent, the sheets were numbered 1 through to 3. The orientation of the graph paper was noted with an x for the bottom horizontal edge of the graph paper and y for the left vertical edge of the graph paper.

Analysis of the images was carried out via Photoshop CS4. The images were adjusted to maximise contrast between the phenolphthalein drops and the graph paper. The results were analysed by reviewing the pattern in a randomly selected square of graph paper. The random selection was achieved using three random number columns created in Excel. The first column contained randomly selected numbers from 1 to 3. This column related to the sheet number for each sprayer. The second random number column consisted of numbers from 1 to 10 corresponding to the number of 10mm² squares along the horizontal axis of the graph paper. The final column contained randomly selected numbers from 1 to 15 which related to the number of 10mm² squares along the vertical axis of the graph paper. 10 samples were analysed for each sprayer.

The samples consisted of one 10mm² square with each sample defined and chosen via three numbers taken from each of the random number columns. For example, a reference number of 3:6:10 relates to graph sheet sample 3, x coordinate number 6 and y coordinate 10. Once chosen, the number of 1mm² covered and not covered with phenolphthalein solution in the given 10mm² sample area were counted. This was used to assess the spray density and coverage that each sprayer gives. To assess droplet size, the number of 1mm² squares, which each droplet in a given 10mm² sample area covered, were counted as well as the number of droplets in the same area. This was used to assess the average size and number of droplets for each sprayer type.

3.6 Morphoanalytical Studies

Spraying luminol onto blood patterns on non-porous surfaces usually results in blood pattern distortion or destruction because the oxidizing agents produced by the

reaction between luminol and the blood are not soluble. Therefore, the can react with luminol molecules and cause chemiluminescence away from where the original blood stain was.

Aim: To evaluate the change in morphology of blood stains treated with latent blood detecting reagents both qualitatively and semi-quantitatively by assessing the ability of different reagents, application methods and fixatives to resolve blood patterns on non-porous surfaces.

The morphoanalytical studies were split into three main sections to see which method most effectively reduces the destruction to blood patterns sprayed with latent blood detecting reagents as described in section 2.4. The first study was to determine if any of the reagents utilised in this thesis reduce the amount of destruction to bloodstains. The second study investigated whether different application methods can be used to reduce the destructiveness of luminol to bloodstains. The final study examined the use of fixatives or shear thinning agents with luminol to reduce the destructiveness to blood patterns. Assessment was based on each methods ability to retain the dimensions of different sized bloody lines on non-porous vertical and horizontal surfaces. Thus on vertical surfaces, the extent of "running" was evaluated and on horizontal surfaces, the extent of "pooling".

For each of the three main sections blood was diluted to 1:1000. This dilution was chosen as it best represents the concentration of blood that would be invisible to the unaided eye. It also will potentially be encountered at a crime scene and enhanced with luminol. Additionally, at higher dilutions and with whole blood, the luminol only reacted with the edge of the lines leaving the middle blank. At lower dilutions, the blood tends to spread more which would interfere with the resolution of the lines. Also, at this dilution the light obtained from the reaction between the bloodstains and luminol is sufficiently bright to be easily seen so as to not loose potentially important data.

A Vikron cleaned, wide rimmed, and shallow, glass dish half filled with 1:1000 diluted blood. Calibrated "blood blocks" (refer to section 2.9.4.1) were use to transfer

the diluted blood to the substrate. Three blocks were made to produce lines of 3mm, 2mm and 1mm, respectively. The lines were separated by troughs of the same thickness for the length of each block. The substrates for this experiment were tiles and vinyl which are described in section 2.3.2 and 2.3.3. First the blocks were partly submerged in the diluted blood till about one 2.5 cm of the blocks was covered.

The blocks were then taken out, dabbed on an absorbent paper towel to mop up excess blood and placed on either a tile or vinyl square. The 3mm block was dabbed once on the paper towel, the 2mm block was dabbed twice while the 1mm block was dabbed three times. The amount of dabbing for each block was predetermined experimentally to achieve the best resolution between the blood lines for each block as the blood spreads to a certain extent.

The samples were left over night to be utilised in experimental work the next day.

For each of the main sections, two separate experiments were undertaken. One assessed how each method affected blood lines on a horizontal non-porous surface and the other assessed how each method affected blood lines on a vertical, non-porous surface. Experiments pertaining to blood lines on a horizontal surface were set up as displayed in figure 3.3 and experiments pertaining to blood lines on a vertical surface were set up as displayed in figure 3.4. As shown in both experiments, the samples and camera were in fixed positions. Three tiles were used for each of the three block types for the horizontal experiments and two tiles and one piece of vinyl were used for the vertical experiments. This was repeated for each variable in the following experiments.

Figure 3.3: Horizontal setup for morphoanalytical experiment

Figure 3.4: Vertical setup for morphoanalytical experiments

Analysis of the experiments pertaining to vertical surfaces was subjectively evaluated based on whether the luminol or fluorescein reactions retained the blood patterns or not. Several categories were esablished to evaluate this. The first category established that the reagent did not retain the blood pattern leading to 'running' and thus the lines could not be resolved. The second category encompassed results where the lines could be resolved but the reagent still ran. The third category encompassed results where the lines could be resolved and the reagent did not run. This last category of course was the desired outcome of the experiments.

Analysis of the experiments pertaining to horizontal surfaces was semi-quantitatively evaluated based on measurement of the troughs and bloody lines before and after application of the reagent. The difference between these two values served as an estimation of the extent of distortion each reagent made to the blood patterns.

Both before and after treatment images were loaded onto Photoshop CS4. The Photoshop ruler was calibrated to the increments on the right angled rulers contained in the image, as displayed in figure 3.3. The width of each line and trough was then measured based on the Photoshop ruler increments set to measure millimetres. The lines and troughs of different images of the samples stamped with the same block were all measured in the same position.

For the 3mm block, the total height of each image was divided into 4 sections of equal size by axis lines running perpendicular to the blood lines. The measurements were taken where the three innermost axis lines intersected the blood lines and along each axis line to measure the width of the blood lines and troughs. For the 2mm and 1mm blocks, the images were divided into three sections of equal size and the two innermost axis lines were utilised to ensure measurements were taken from the same places in each image. The measurements at each intersecting axis line for each line and trough in each image and for each block were recorded in an Excel spreadsheet.

3.6.1 Reagent type

The reagents evaluated were Grodsky's luminol, Bluestar Magnum, Lumiscene, Lumiscene Ultra and Hemascein. Descriptions of these reagents can be found in section 2.4.

Only the vertical experiment was undertaken for the reagent type morphological study. For the vertical experiments a total of 6 tiles and 3 pieces of vinyl were utilised for each reagent, two tiles and 1 piece of vinyl for each of the three blood blocks. Each reagent was sprayed at a fixed distance away via the spray gun. The spray gun was utilised for the delivery of the reagents because it gives a calibrated delivery of each reagent so therefore a known amount of each reagent is delivered to each sample.

However, the problem with using the airgun for experimental work is that the spray mist delivered is so fine that a sprayed blood pattern on a non-porous surface retains detail somewhat well. This poses a problem when trying to assess the

differences in blood pattern destruction for the different reagents. Therefore, I decided to deliberately and equally overspray each sample by spraying for 3 seconds. This time was found experimentally to be favourable when using Grodsky luminol as the pattern starts to run or pool, but does not run or pool excessively.

3.6.2 Application Method

The application methods under evaluation were a common hand pump sprayer, ABA spray, ABA finger spray, ECO spray and a nitrogen powered spray gun. Descriptions of these sprayers can be found in section 2.5

The horizontal and vertical experiments for this experiment were carried out by the same process described in the reagent type experiment. Grodsky luminol was the reagent utilised for this experiment. The distance and time each sample was sprayed depended on the type of sprayer assigned to that sample. Because each sprayer type sprays luminol at a different rate, meaning some samples would receive more reagent than others, it was deemed prudent to spray samples with no fixed time limit. Also, some of the sprayers had a longer range than the others which would also affect the amount of reagent reaching the target. Therefore each sample was sprayed with an assigned sprayer for a length of time and at a distance deemed enough to generate sufficient light to capture an image of the reaction.

3.6.3 Fixatives and Shear thinning agents

The fixatives under examination were two zinc formulas adapted from the work of Lykidis et al (117). The first zinc fixative consisted of 0.5% zinc acetate, 0.05% zinc chloride and 0.5% calcium acetate in distilled sterile water. The second zinc fixative consisted of 0.5% zinc trifluoroacetate, 0.05% zinc chloride and 0.5% calcium acetate in distilled sterile water. The next type of fixative was an alcohol based fixative consisting of a 70:30 acetone: methanol mix. The next potential additives to the luminol formula consisted of shear thinning agents. Both shear thinning agents evaluated in this thesis consisted of xanthan gum. One of these xanthan gum based shear thinning agents is termed ABA fix and manufactured by ABA diagnostics and is combined with their fluorescein based formulation, Hemascein. The other xanthan gum shear thinning agent was obtained from Sigma Aldrich. These concentrations

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were predetermined experimentally to be the best for the purposes of fixing blood for luminol delivery.

The horizontal and vertical experiments for this experiment were carried out by the same process described in the reagent type experiment. Grodsky luminol was the reagent utilised for this experiment. For the alcohol fixative and zinc fixatives, the fixative was first sprayed onto the sample using the ABA finger pump sprayer. The samples were then dried with a hair dryer and placed into position under the camera. Delivery of the luminol was via the spray gun delivered at a fixed distance and at a fixed time of three seconds.

For the ABA fix and xanthan gum shear thinning agents, measured portions of these agents were mixed with the luminol prior to spraying. Ensuring the solution was efficiently mixed, the solution was then poured into the spray gun reservoir. The samples were then in turn placed under the camera and sprayed with the luminol using the spray gun at a fixed distance and a time interval of three seconds.

A combination of the zinc 2 fixative with the xanthan gum was then added to the experiment. The zinc fixative was first sprayed onto the sample with the ABA finger pump sprayer and dried with a hair dryer. The sample was then place in position under the camera and sprayed with the luminol/xanthan gum mix via the ABA spray. This was repeated with the ECO spray. The ECO spray was utilised as this sprayer is more efficient in the delivery of the luminol/xanthan gum mix than the spray gun as the spray pathway has a tendency to become obstructed by the gum. The ABA spray was utilised as a comparison to the ECO spray as the two sprayers operate via different mechanisms.

4. Results

4.1.1 Quantitative analysis

The first part of this experiment sought to quantitatively differentiate between the latent blood detecting agents based on their sensitivity to blood. Data output from the photographs of the different reagents reaction with blood at different dilutions, analysed via Photoshop and Matlab, was compiled onto an EXCEL spreadsheet.

Below are scatter plots for each of the different reagents showing the relationship between light intensity of the reactions and blood concentration. Light intensity is plotted in arbitrary units as designated by Matlab software. Blood concentration is plotted as a dilution factor in log scale. For Grodksy, Bluestar Magnum, Lumiscene and Lumiscene Ultra the light intensity includes only the blue channel for this was the dominant channel. For Hemascein only the green channel intensity is plotted.
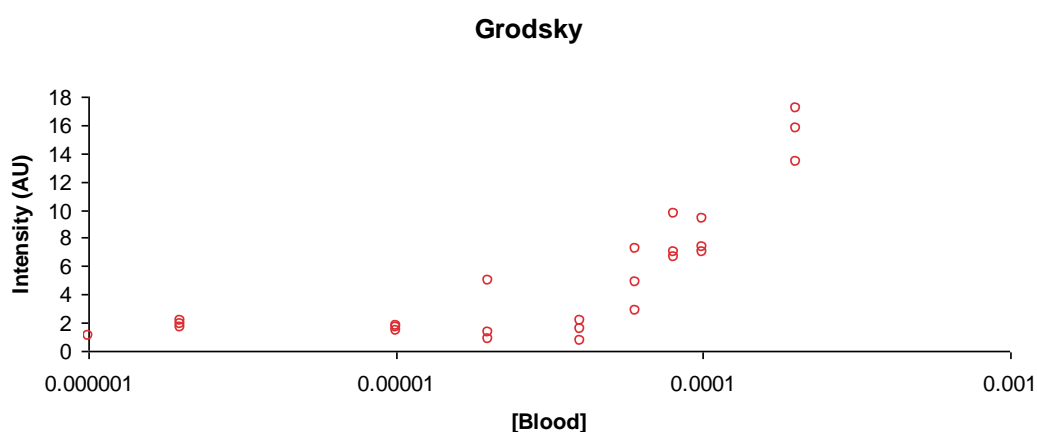
Figure 4.1: Grodsky Chemiluminescence Light Intensity vs. Blood Concentration
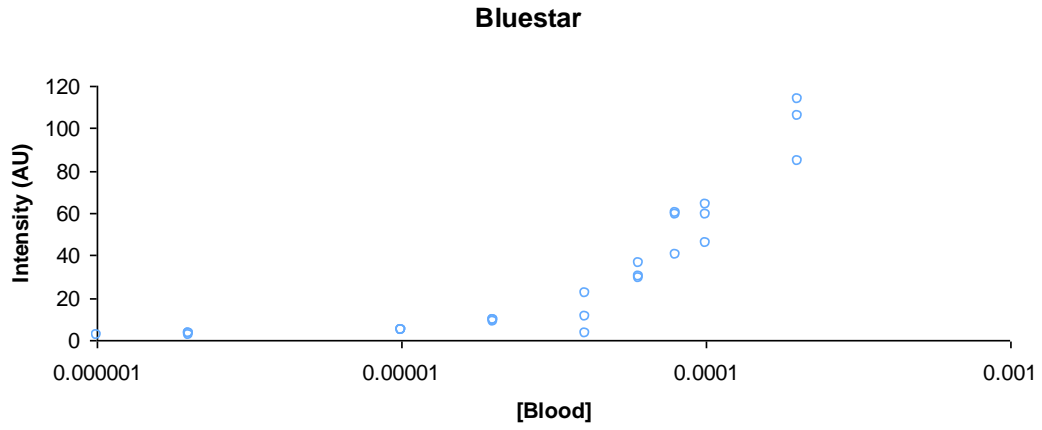
Bluestar

Figure 4.2 Bluestar Chemiluminescence Light Intensity vs. Blood Concentration
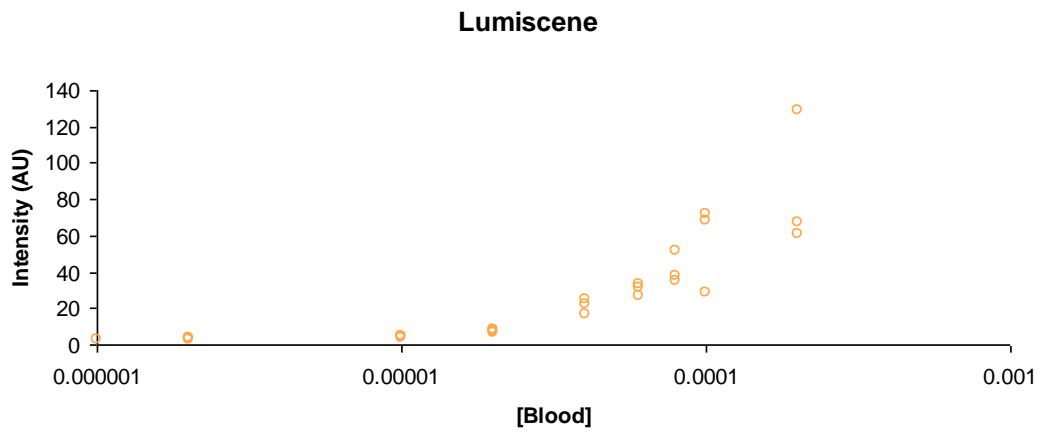
Lumiscene

Figure 4.3 Lumiscene Chemiluminescence Light Intensity vs. Blood Concentration
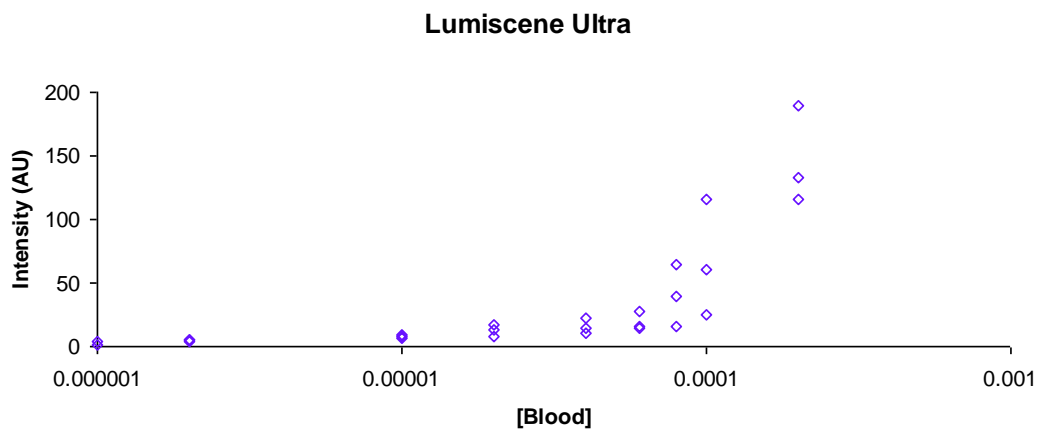
Lumiscene Ultra

Figure 4.4 Lumiscene Ultra Chemiluminescence Light Intensity vs. Blood Concentration
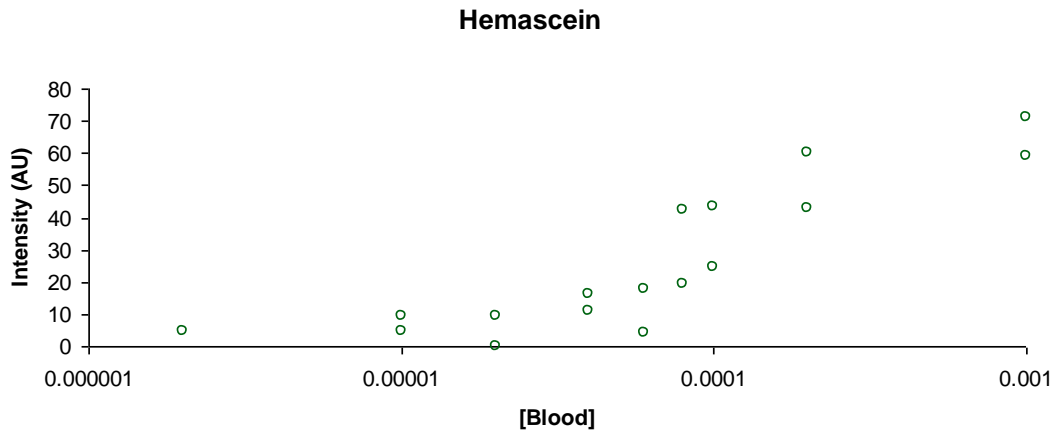
Hemascein

Figure 4.5 Hemascein Fluorescent Light Intensity vs. Blood Concentration

The above graphs show a decrease in the light intensity of the reaction with decreasing blood dilutions. Also apparent is the increasing variance toward higher blood concentration. The data also contains no outliers. In these graphs it appears that a linear model is an inadequate expression of the data. Linear regression plots (Appendix) show the smiler pattern, confirming this observation.

All the reagents average intensity values were then plotted onto a single graph for comparative purposes. For simplicity, error bars are omitted.
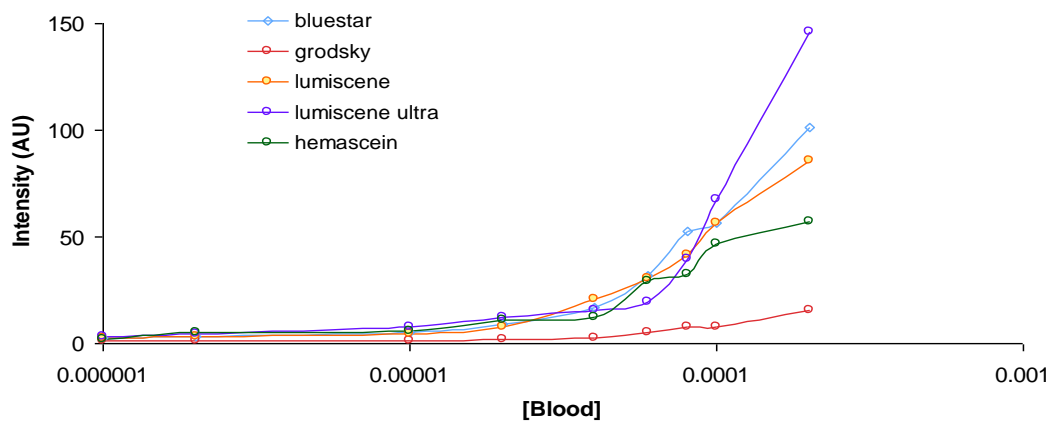
Figure 4.6 Graph comparing the average values of light intensity vs. blood concentration for the five latent blood detecting reagents.

From this graph, Bluestar magnum, Hemascein, Lumiscene and Lumiscene Ultra all appear to have intensity values higher to a greater extent than that of Grodsky until

the lower three dilution values. Lumiscene Ultra appears to have a much higher intensity value at the highest blood dilution value (1:5000) though quickly drops intensity in the subsequent blood dilution values. Bluestar Magnum and Lumiscene seem to have similar intensity values throughout the dilution series.

Multiple regression was then undertaken to statistically show whether one reagent out performs the other. Examination of the figures shows that blood concentration, as shown by the previous graphs, has a significant effect. To eliminate this effect of concentration each reagent was tested against each other using multiple regression. Below is a table giving the results.

	Lumiscene Ultra	Lumiscene	Grodsky	Hemascein
Bluestar magnum	0.293	0.454	9.5E-08	0.0753
Lumiscene Ultra		0.177	3.3E-05	0.0978
Lumiscene			6.07E-07	0.357
Grodsky				8.25E-8

Table 4.1 The p-values for the multiple regression testing each reagent against the others.

The p-values show that Lumiscene Ultra, Bluestar Magnum, Hemascein and Lumiscene performed significantly better than Grodsky. However, Lumiscene Ultra, Lumiscene, Hemascein and Bluestar Magnum could not be statistically separated based on their p-values. The average intensity value for Lumiscene Ultra exceeded that of Bluestar Magnum which was higher than Lumiscene which in turn was higher than Hemascein, although not significantly.

4.1.2 Qualitative analysis

The second part of this experiment was to evaluate the sensitivity qualitatively. This was assessed on two levels. The first level was to assess at which dilution a positive reaction can still be seen by with the unaided eye and with unenhanced images of the reaction. The second level involved computer enhancement whereby Photoshop enhanced images were then processed through Matlab software. Matlab code for image processing in this thesis was written by Dr Gordon Miskely of the Chemistry department at The University of Auckland and is discussed in detail in section 3.2.1.

Photoshop enhancement involved adjusting the contrast, colour and brightness of the images.

Samples were created in triplicate for Lumiscene Ultra, Lumiscene, Grodsky and Bluestar Magnum and duplicate for Hemascien at each of the 10 dilutions. The following images are the best representations of each reagents reaction at the given dilution factor out of the repeats. The left hand column images are the images where the reaction is visible, the middle column contains images where the reaction is barely visible and the third column contains images where the visibility of the reaction is questionable. The first row of each image block consists of images where no modification has been done to the image; the second row are un-averaged images processed in Matlab to the 3x3 filter stage before geometric averaging; and the final row consists of images where the image of the reaction has been geometrically averaged using Matlab.

Grodsky

1:5000   1:10,000   8:100,000

6:100,000   4:100,000   2:100,000
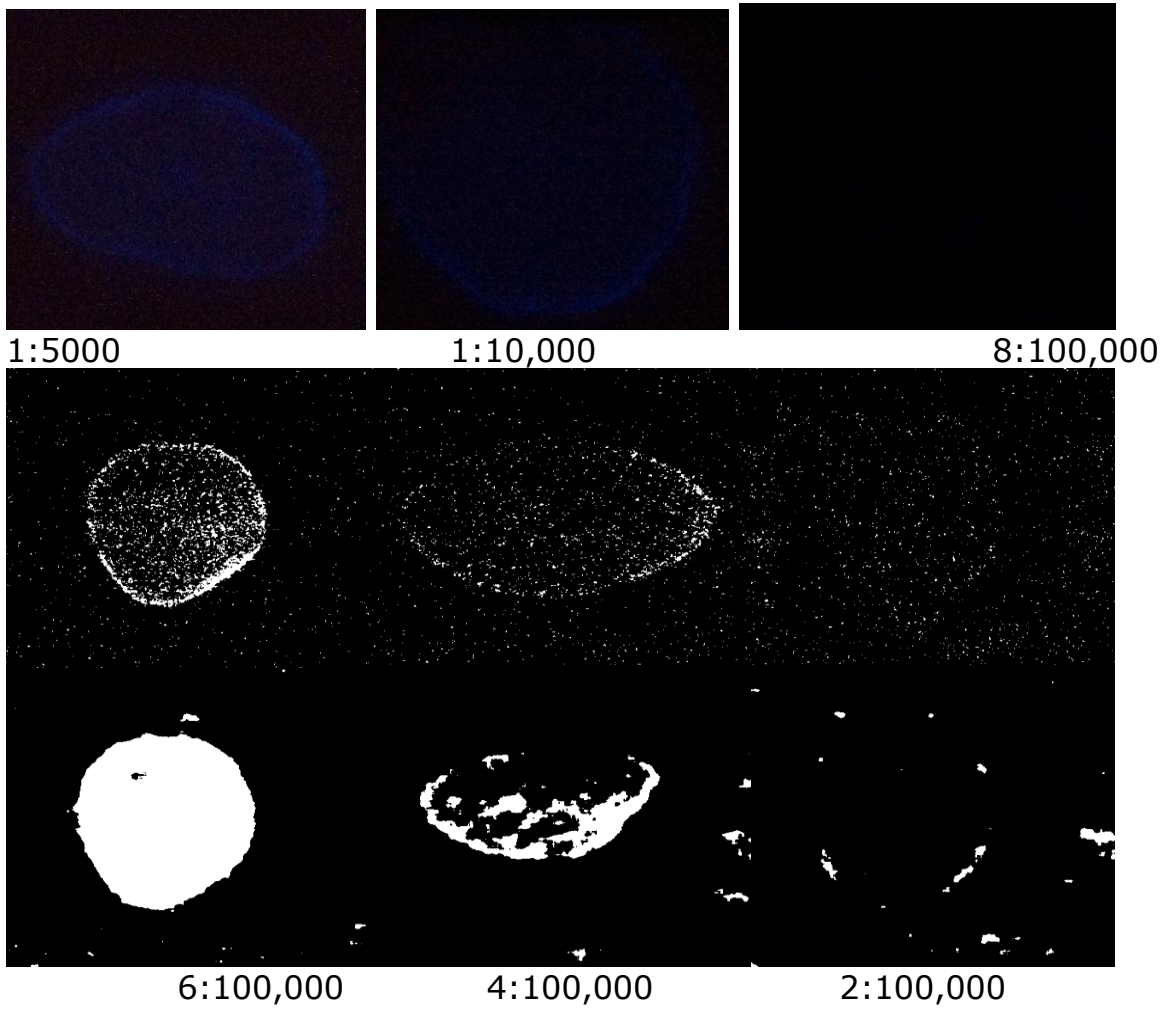
Figure 4.7: Grodsky images for the sensitivity experiment

Bluestar Magnum

6:100,000   4:100,000   2:100,000
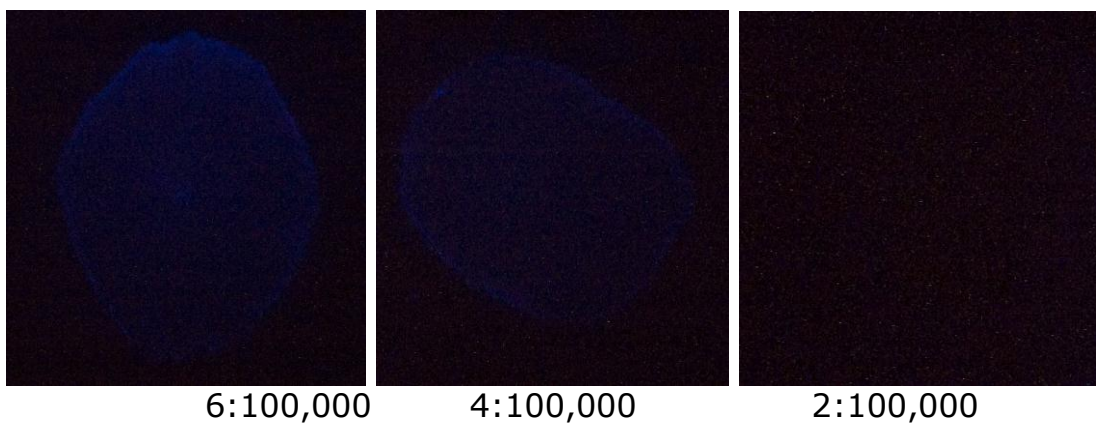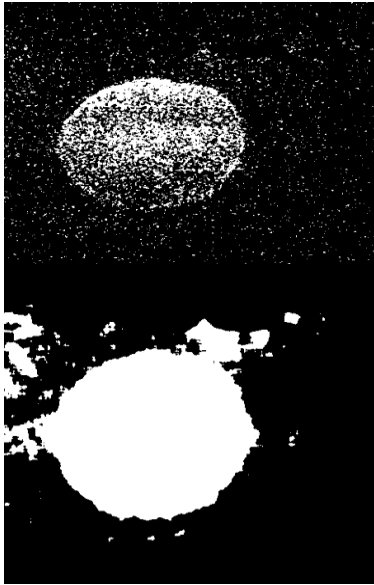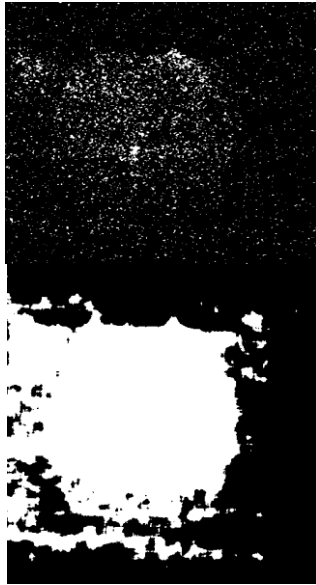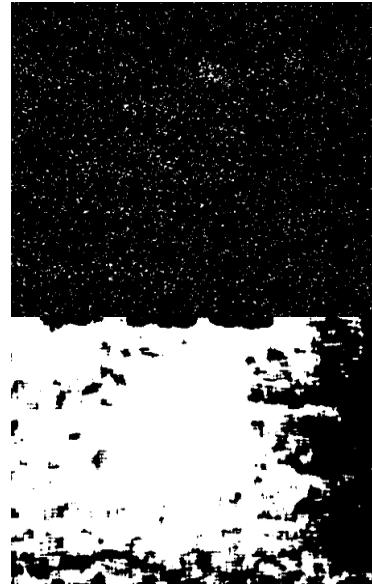
2:100,000   1:100,000   1:500,000

Figure 4.8: Bluestar Magnum Images for Sensitivity Experiment

Lumiscene

6:100,000   4:100,000 2:100,000

2:100,000   1:100,000   1:500,000
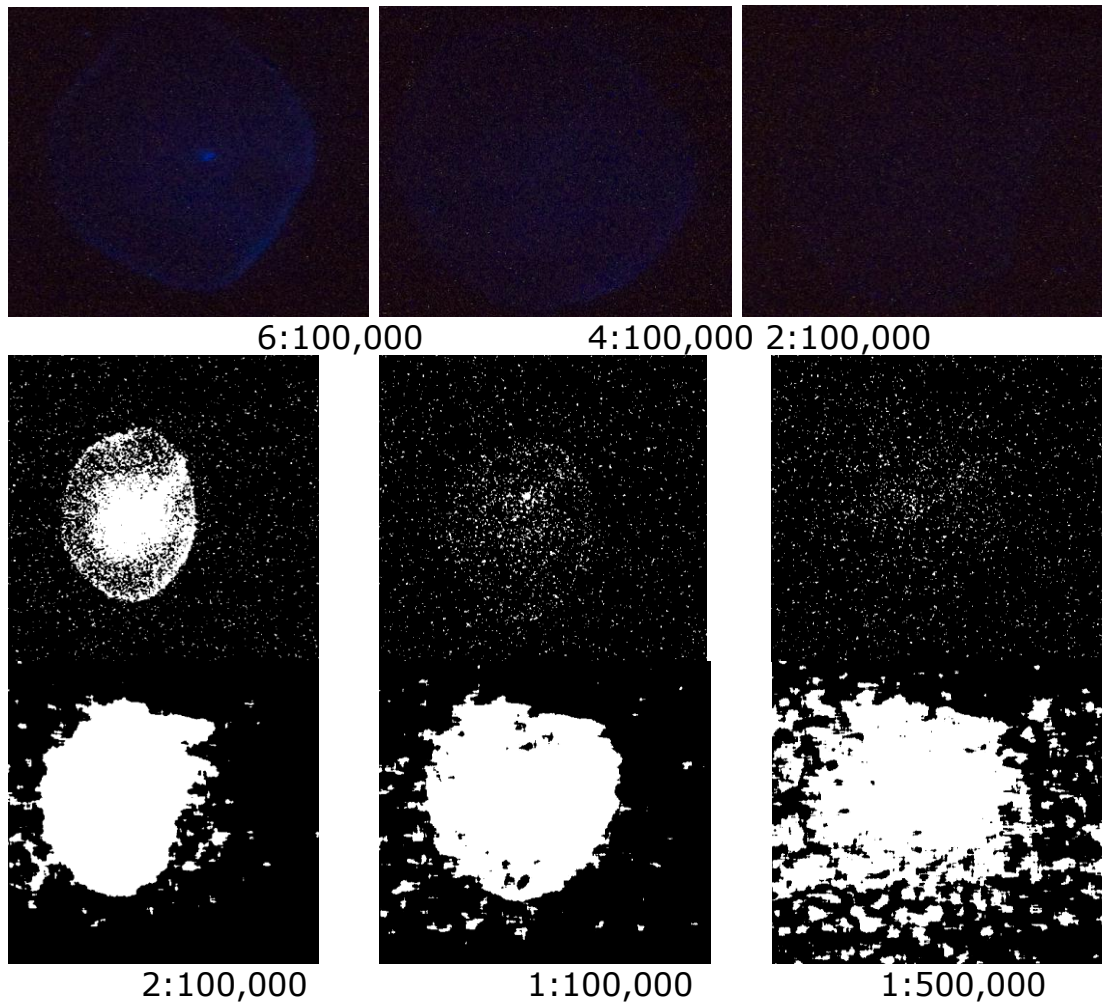
Figure 4.9: Lumiscene Images for Sensitivity Experiment

Lumiscene Ultra

| 6:100,000 | 4:100,000 | 2:100,000 |

| 2:100,000 | 1:100,000 | 1:500,000 |
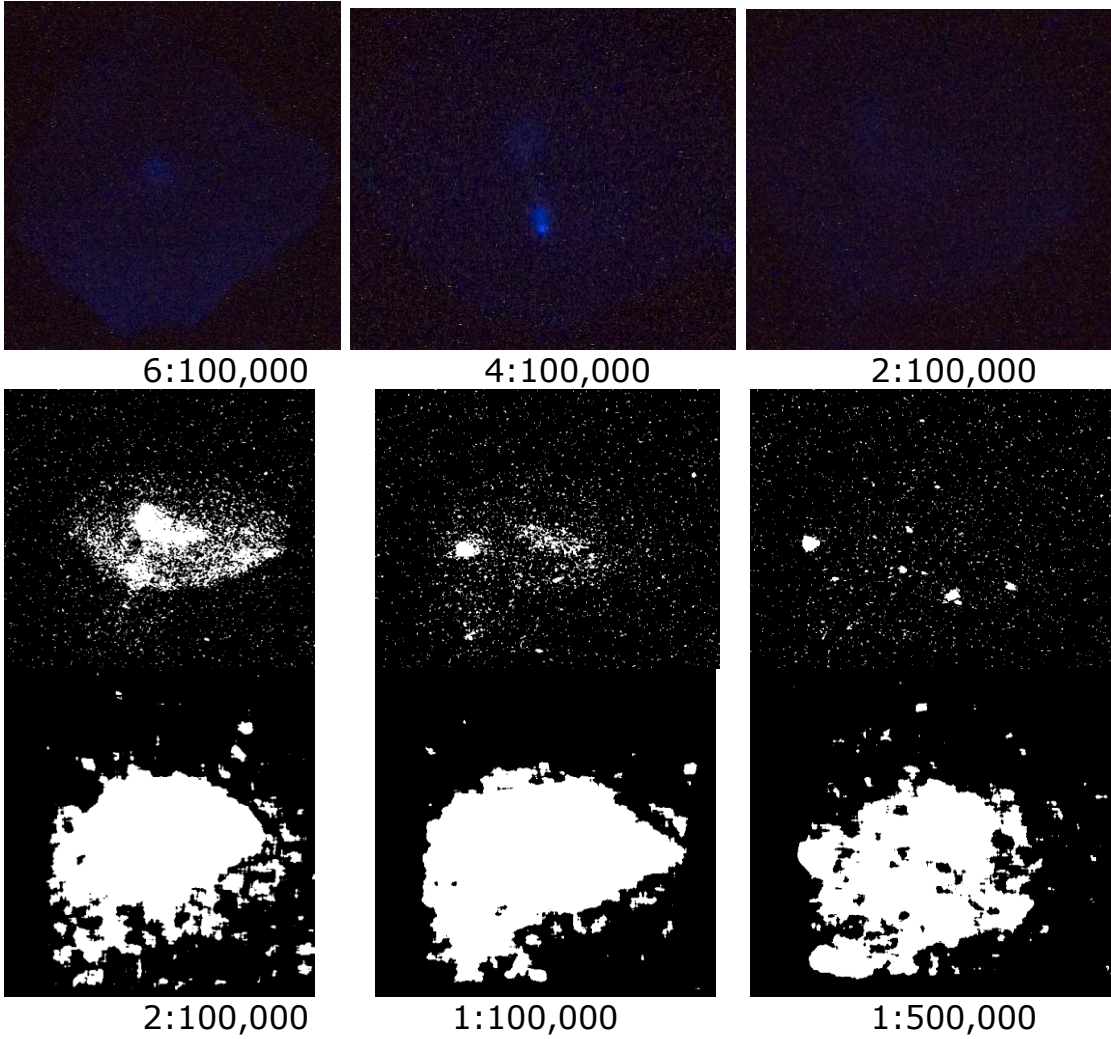
Figure 4.10: Lumiscene Ultra Images for Sensitivity Experiment

Hemascein

6:100,000   4:100,000   2:100,000

6:100,000   4:100,000 2:100,000

4:100,000

2:100,000

1:100,000
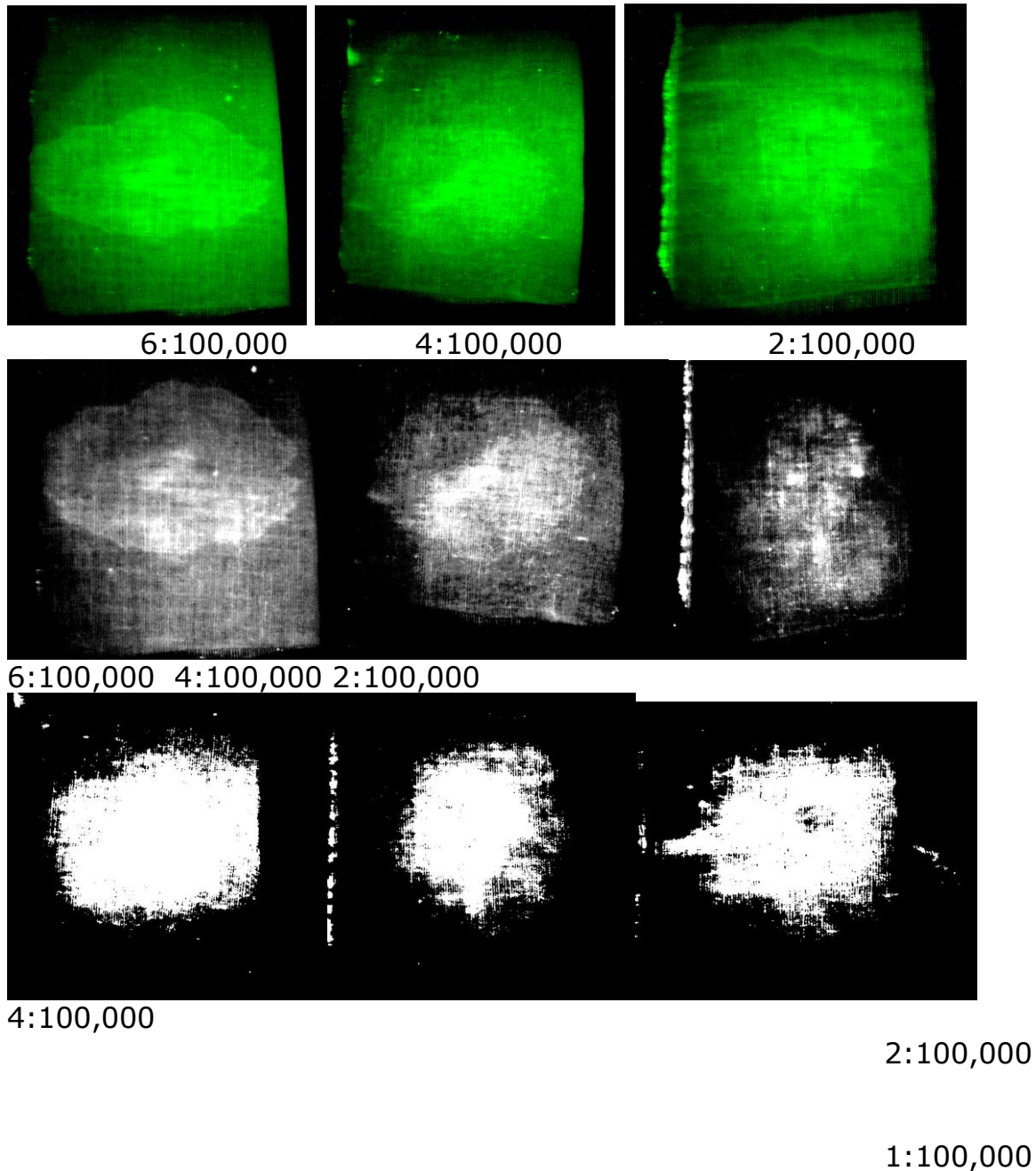
Figure 4.11: Hemascein Images for Sensitivity Experiment

Below is a table summarising the images above. The Upper limit relates to reactions which could be easily seen and the lower limit relates to reactions that are not as obvious.

	Visual	Matlab Unaveraged	Matlab-Averaged		Visual
	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit

Grodsky	1:5000	1:10,000	6:100,000	4:100,000	6:100,000
Bluestar Magnum	6:100,000	4:100,000	2:100,000	1:100,000	2:100,000

Lumiscene	6:100,000	4:100,000	2:100,000	1:100,000	2:100,000	1:100,000
Lumiscene Ultra	6:100,000	4:100,000	2:100,000	1:100,000	2:100,000	1:100,000
Hemascein	6:100,000	4:100,000	4:100,000	2:100,000	-	-

Table 4.2 Showing the results of the qualitative sensitivity study

From the images and table above it can be observed that all the reagents can detect lower blood concentrations than Grodsky. In the visual column, Bluestar Magnum, Lumiscene, Lumiscene Ultra and Hemascein have the same limits of detection. For the Matlab un-averaged values, Lumiscene, Lumiscene Ultra and Bluestar detected lower concentrations than hemascein. For the Matlab averaged values, Lumiscene Ultra appeared to have the lowest detection limit followed by Lumiscene and Bluestar. No values were recorded for Hemascein because of background interference. The background cloth gave a false positive result when averaging was attempted which gave irregular images unrelated to the hemascein reaction with the bloodstain.

4.1.3 Limit of Detection Estimation

Based on the results obtained from both the quantitative and qualitative studies of the different reagents sensitivity to blood, a tentative limit of detection can be defined. The upper limit of detection was chosen at 10 AU and related to the amount of light emitted from a reaction which can reliably be seen with the unaided eye. The lower limit of detection was chosen at 5 AU and related to the amount of light emitted from a reaction which can reliably be visualised through computer enhancement. The figures listed below are for comparative purposes, not absolute figures. Relative intensity is scaled in log units.
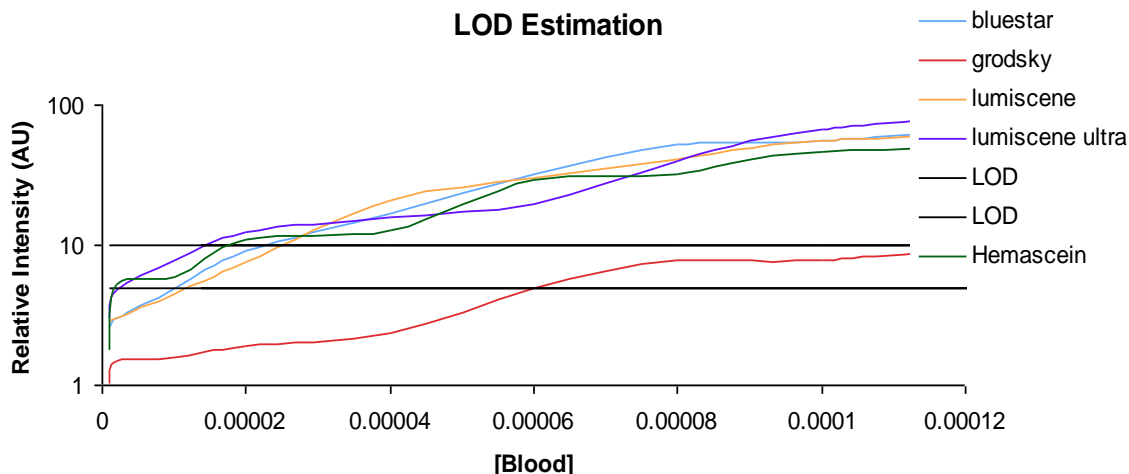
Figure 4.12: Graph showing the relative intensity vs. blood concentration and an estimation of the limit of detection for each of the reagents.

The following table contains the LOD values for each reagent based on the above graph. The values for the LODs were assigned by selecting the concentration value pertaining to the minimum concentration allowing successful detection of the positive reaction. Close calls, where the LOD line crossed the reagents line just before or just after a concentration value, were judged by whether the reaction could be seen in the images.

	Grodsky	Bluestar Magnum	Lumiscene	Lumiscene Ultra	Hemascein
LOD 10 AU	1:5000	4:100,000	4:100,000	2;100,000	4:100,000
LOD 5 AU	6:100,000	2:100,000	2:100,000	1:100,000	2:100;000

Table 4.3: Shows the limit of detection for each of the reagents at 10 and 5 intensity units.

From the above table it can be seen that Grodsky is the least sensitive reagent. Bluestar, Hemascein and Lumiscene have a similar LOD at both intensities while Lumiscene Ultra proved to be the most sensitive.

4.2 Longevity of the Reaction

4.2.1 Quantitative Analysis

The first part of this experiment sought to quantitatively differentiate between the latent blood detecting agents based on their reaction with blood at 1:1000 dilution over time.

Data output from the photographs of the different reagents reaction with blood at different dilutions, analysed via Photoshop and Matlab, was compiled into an EXCEL spreadsheet

The graph below shows the average intensity values for the reagents at each time interval measured from 0 to 5 mins. With the luminol based reagents, photographs were taken every 30s with long exposure times of 30s. Therefore, the value at time 0.5 minutes is the intensity of the light received by the camera during the first 30s of the reaction, and so on. For Hemascein, photographs were taken every 10 seconds for reasons stated in section 3.3. Time is plotted on the x axis in minutes and the log of light intensity is plotted on the y axis in arbitrary units as designated by Matlab software.
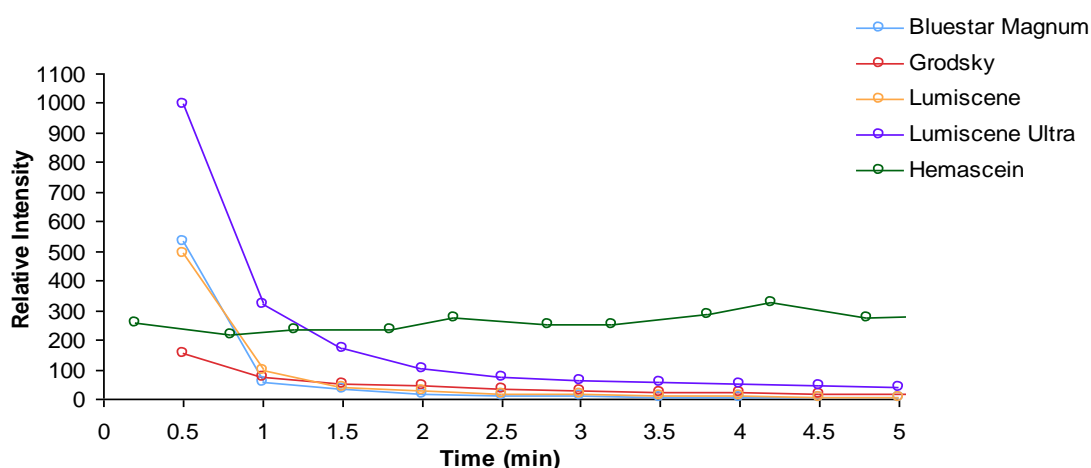
Figure 4.13: Showing the intensity values first 5 minutes of each of the reagents reactions

From this graph it is evident that for Bluestar Magnum, Lumiscene, Lumiscene Ultra and Grodsky the intensity of the reaction quickly decreases as time progresses. For these reagents the biggest drop in this graph appears to be between 0 and 1 relating to the first minute of the reaction. From this point on, the intensity appears to decrease slowly to lower intensities as time progresses. Heamascein is different from the other reagents in this graph, which is not inconceivable because hemascein is fluorescein based while the other reagents are luminol based. The intensity of the Heamscein reaction with blood appears overall to slightly increase over the first 5 minutes of the reaction rather than dramatically decrease as with the other reagents.

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Examination of the figures shows that time for the luminol based reagents, not unexpectedly, has a significant effect. A question of interest is whether one method out performs the other. Each reagent was tested against each other using multiple regression to eliminate the accepted effect of time. Below is a table giving the results

	Lumiscene Ultra	Lumiscene	Grodsky	Hemascein
Bluestar Magnum	0.0370	0.914	0.498	4.33E-7
Lumiscene Ultra		0.0393	0.00894	0.0190
Lumiscene			0.375	9.64E-11
Grodsky				1.78E-9

Table 4.4: The *p*-values for the multiple regression testing each reagent against the others

LU had a significantly better performance in experiment 2 than all of the other reagents. Lumiscene, Grodsky and Bluestar could not be statistically separated based on these results. However this could be due to the massive difference in relative intensity of the initial reading at time 0-0.30s. Taking this value out for Lumiscene, Grodsky and Bluestar and following with multiple regression shows *p*-values of 0.000444 for Grodsky and Bluestar and 0.169 for Grodsky and Lumiscene. In both cases the t stat was positive for Grodsky meaning the mean relative intensity for Grodsky is significantly higher than that of Bluestar magnum but not significantly higher than Lumiscene. Below is a table summarising this.

	Bluestar Magnum	Lumiscene
Grodsky	0.000444	0.169

Table 4.5: The *p*-values for the multiple regression testing Grodsky against Bluestar Magnum and Lumiscene without the first value (0.5 minutes).

Taking the log of intensity for the graph in figure 4.13 emphasises the differences between each of the reagents. This graph is plotted below.
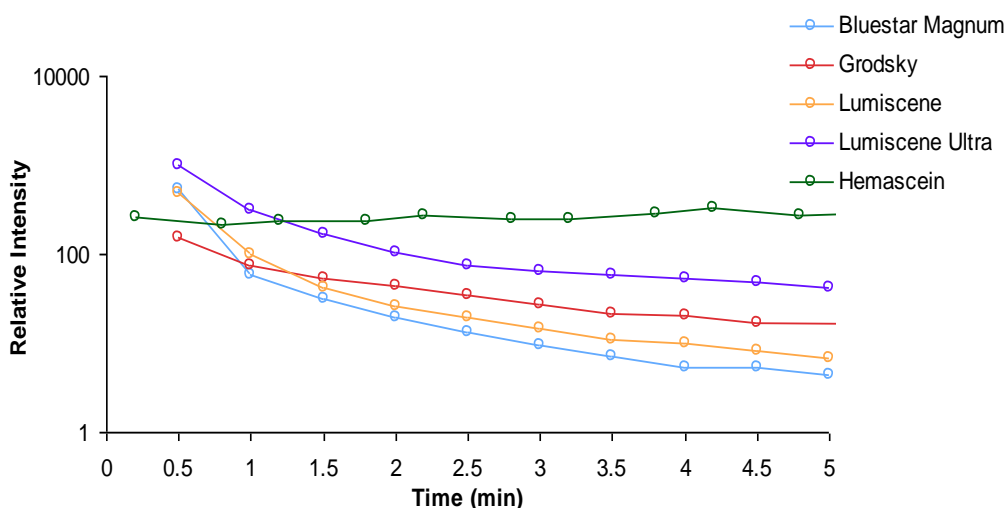
Figure 4.14: Graph showing log intensity for the first 5 minutes of each of the reagents reactions

Examination of the graph shows the decay of chemilumiescene seems fast in Bluestar and Lumiscene due to the slope of the graph when compared to Grodsky and Lumiscene Ultra where the slope is gentler corresponding to slower chemilumiescent decay. The residual plots for all the luminol based reagents show the smiler pattern which suggests a linear model is not an adequate description of the data. However, if the first or first couple of plots are ignored then a linear relationship between chemiluminescent decay and time can be observed up to 5 minutes of the reaction. From the residual plots of Hemascein, a linear relationship between chemiluminescent decay and time was observed up to 5 minutes of the reaction.

For interest, the reactions were then additionally continued for 15 more minutes with photographs taken at 5 minute intervals. The average intensity results of this experiment are graphed below. Relative intensity is plotted as log of intensity on the y axis and time in minutes on the x axis
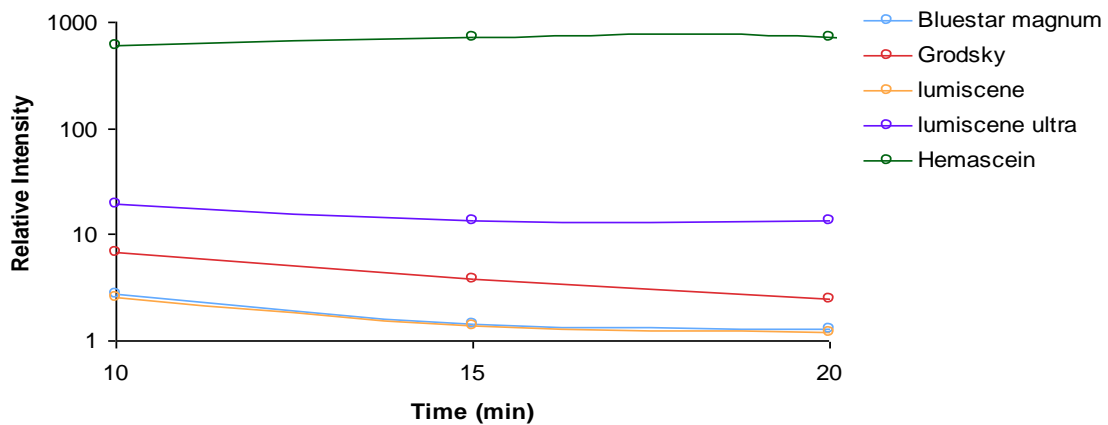
Figure 4.15: Graph showing the intensity of the reaction for each reagent between 10-20 minutes of the reaction

From this graph it is apparent that the intensity of the Hemascein reaction is considerably higher than the others between 10 to 20 minutes after inception. Lumiscene Ultra continues to maintain a higher intensity than the other luminol based reagents. Grodsky, after starting with a much lower intensity a the beginning of the reaction than Bluestar Magnum and Lumiscene (figure 4.15), appears to have higher intensities than both Bluestar Magnum and Lumiscene during this 10-20 minute interval.

The Hemascein reaction with blood is fluorescent which has a significantly greater longevity than the chemiluminescent reaction of luminol. Additional images were captured of the Hemascein reaction up to two and a half hours after the start of the reaction. These intensity values were calculated in Matlab and plotted into the graph below along with the other time intervals to give a profile of the light intensity emitted from the Hemascein reaction as it changes with time. Intensity is plotted in arbitrary units on the y axis and the log of time is plotted on the x axis in minutes.
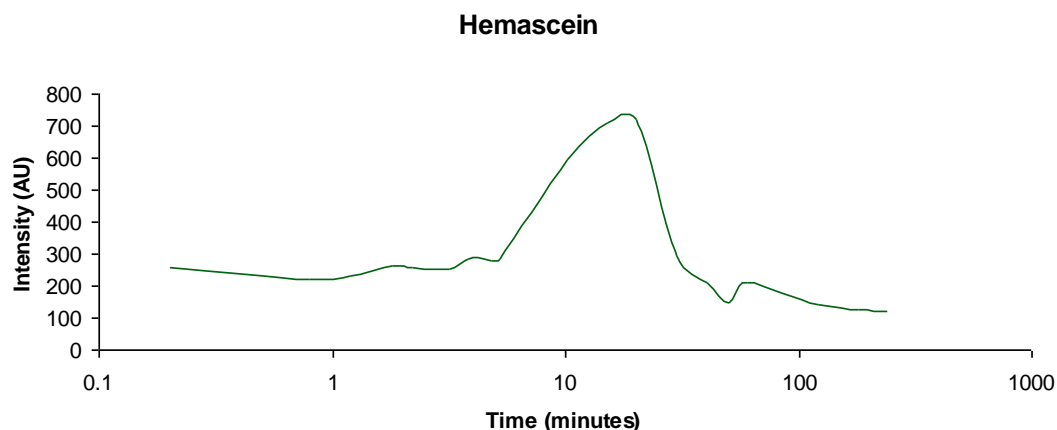
Hemascein

Figure 4.16: Graph of the intensity of the Hemascein reaction changing over time from the start of the reaction till 2.5 hours after.

The shape of this graph is very interesting. The reaction seems fairly constant during the first 5 minutes of the reaction. Between 5 minutes till around 15 to 20 minutes there seems to be an exponential rise in fluorescent intensity quickly followed by an equally quick exponential decay to about 30 minutes after the reaction. This is followed by a further decrease in intensity 40-50 minutes after the reaction, a slight rise in intensity after 60 minutes before a fairly constant decrease from one hour till two and a half hours after the start of the reaction.

4.2.2 Qualitative Analysis

The second part of this experiment involved qualitatively evaluating the longevity of the reactions. Samples for times 0-5 minutes were created in triplicate for each reagent. Samples for times 10-20 minutes were created as singular for each reagent except for hemascein were triplicates were created. A further 7 samples were made for hemascein for times 30 minutes to two and a half hours for one of the samples. The following images are the most representative images for the given reagent and time out of the samples produced.

The left hand column images of the chemiluminescent reactions are the images where the reaction is just started, the middle column contains images where the light from the reaction begins to fade from the middle of the stain and the third column contains images where the visibility of the reaction is just visible. For the Hemascein images only the visual images were shown at varying times.

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The middle of the stain fading creates a "ring effect" where the edges stains have a higher intensity than the rest of the stain. This effect is considerably noticeable in the samples treated with the luminol based reagents, not Hemascein.

The first row of each image block consists of images where no modification has been done to the image, the second row are un-averaged images processed in Matlab to the 3x3 filter stage before geometric averaging and the final row consists of images where the image of the reaction has been geometrically averaged using Matlab. Again, the Hemascein images below are only the visual images shown at varying times.

Grodsky
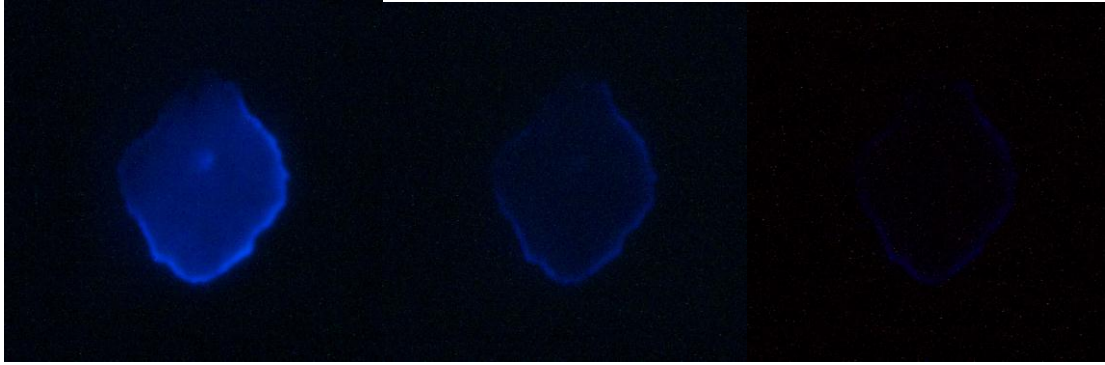
0-0.5mins   2-2.5mins   4.5-5mins
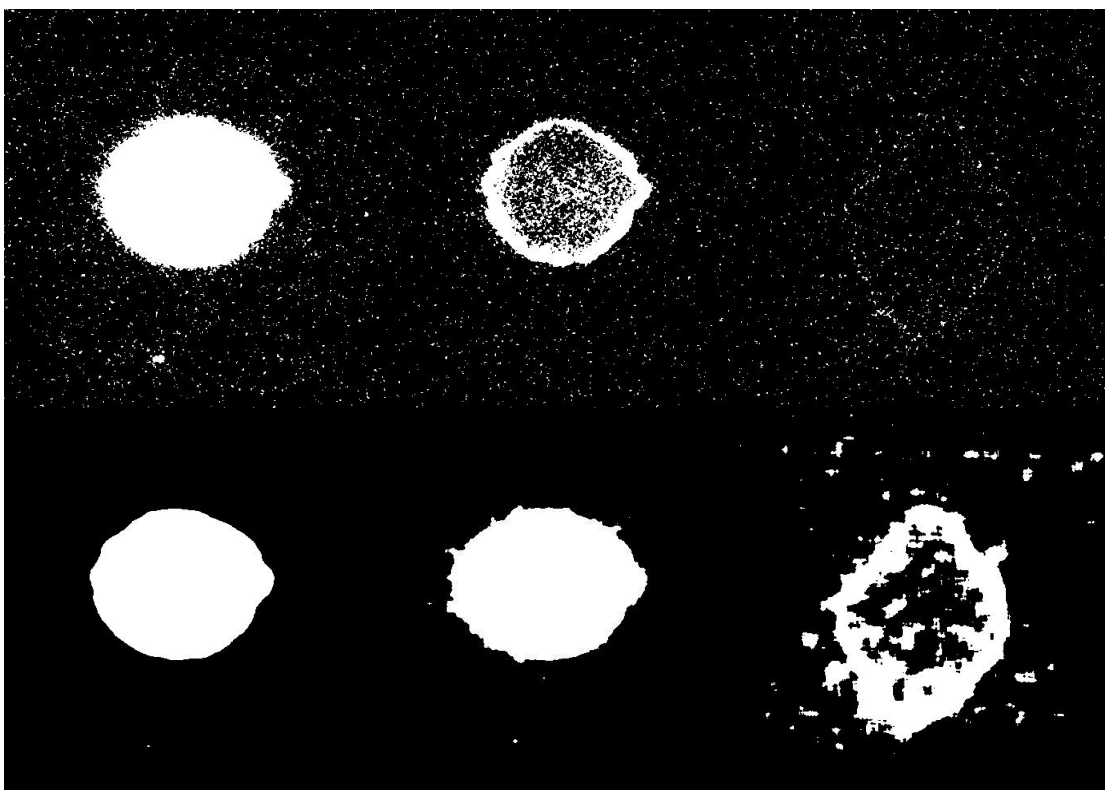
2-2.5mins   4.5-5 mins   20 mins

Figure 4.17 Grodsky Images for Reaction Length Experiment

Bluestar Magnum
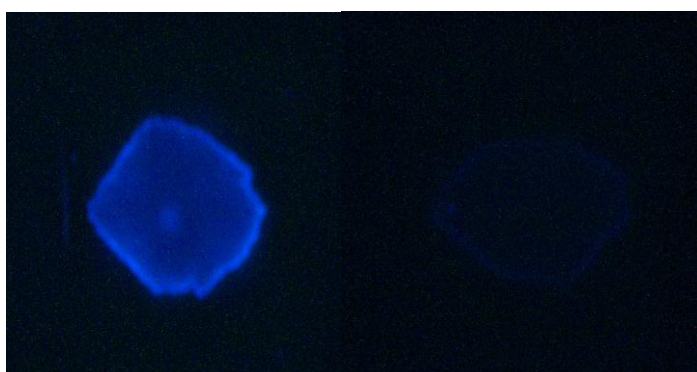
0-0.5mins   2-2.5mins   4.5-5mins
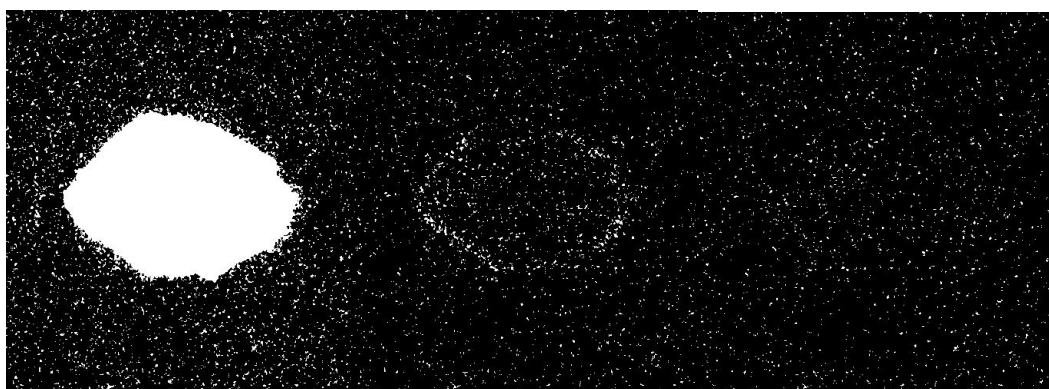
0.5-1 mins   2-2.5mins   4.5-5mins

0.5-1mins   4.5-5mins   10mins

Figure 4.18 Bluestar Magnum Images for Reaction Length Experiment

Lumiscene

0-0.5mins   3-3.5mins   4.5-5mins

0.5-1mins   3-3.5mins   4.5-5mins

0.5-1mins   4.5-5mins   10mins
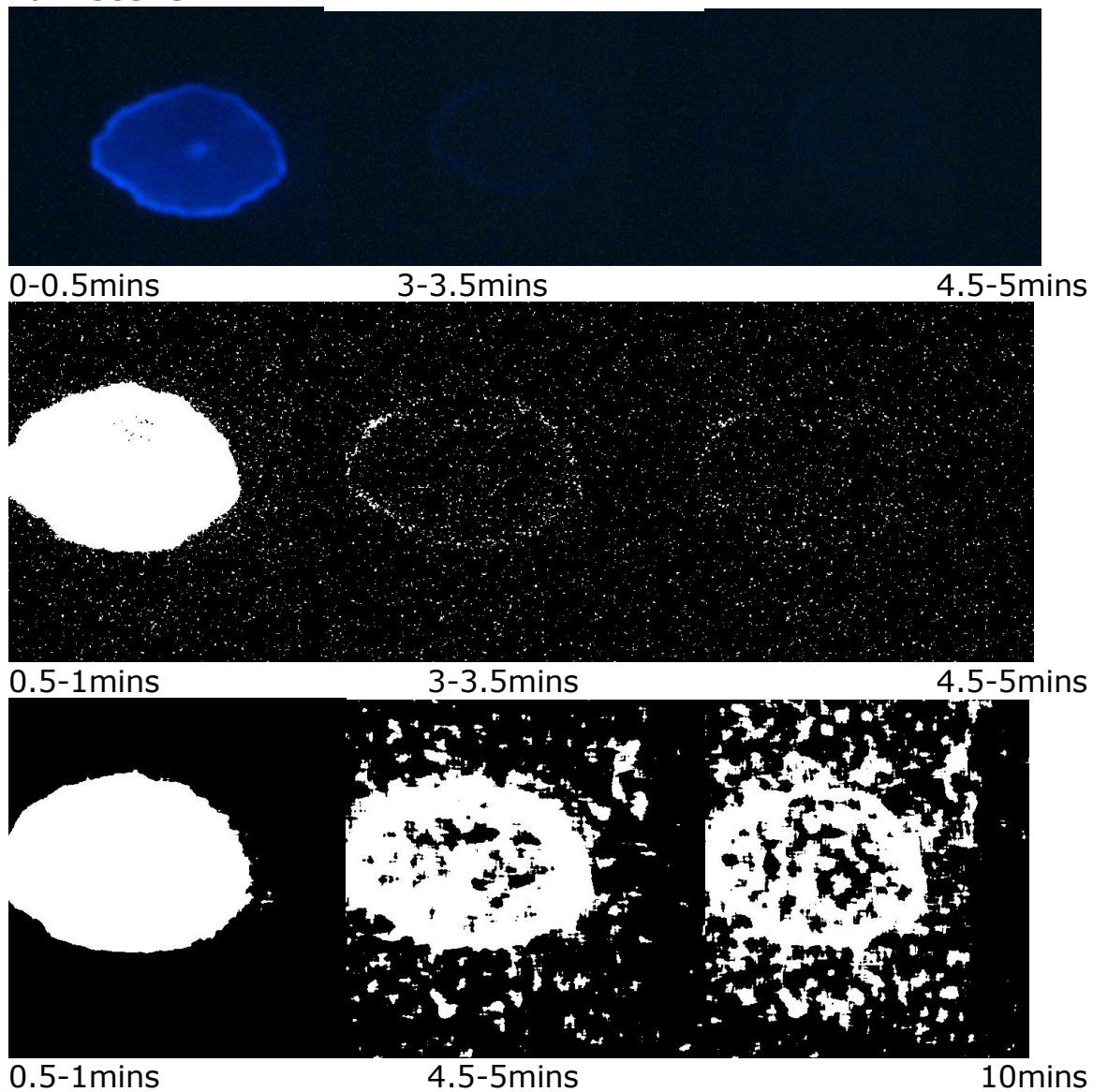
Figure 4.19 Lumiscene Images for Reaction Length Experiment

Lumiscene Ultra

0-0.50mins   2-2.5mins   4.5-5mins

1-1.5mins   4.5-5mins   15mins
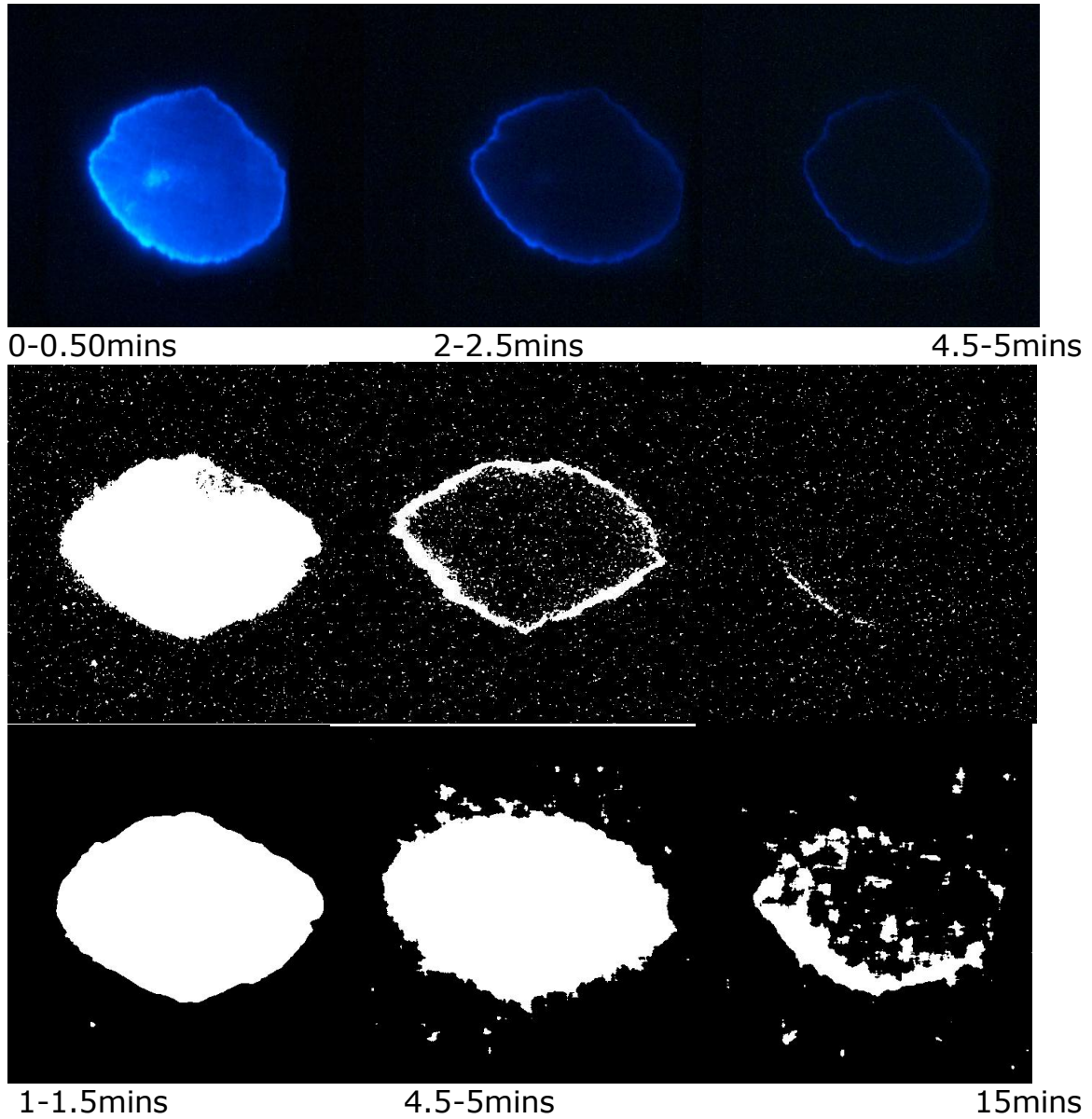
Figure 4.20 Lumiscene Ultra Images for Reaction Length Experiment

Hemascein

0-0.2mins   2-2.2mins   5-5.2mins

20mins   2hrs 30mins
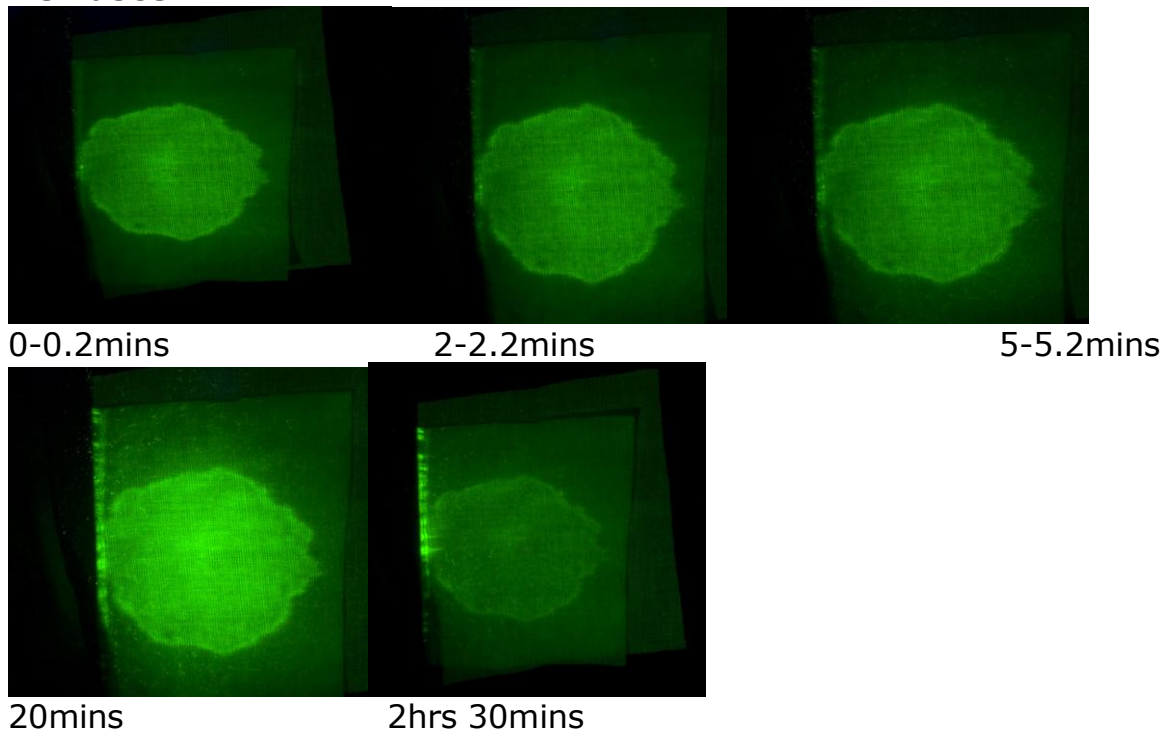
Figure 4.21 Hemascein Images for Reaction Length Experiment

Below is a table summing up the information from the pictures above.

	Visual		Matlab Unaveraged		Matlab- Averaged	
	Upper limit	Ring formation	Upper limit	Ring formation	Upper limit	Ring formation
Grodsky	4.5-5	1.5-2	15	3.5-4	20	15
Bluestar Magnum	2-2.5	0.5-1	2.5-3	1.5-2	10	4.5-5
Lumiscene	2-2.5	0.5-1	2.5-3	1.5-2	10	4-4.5
Lumiscene Ultra	4-4.5	1.5-2	10	2.5-3	15	10
Hemascein	2.5hrs	-	2.5hrs	-	2.5hrs	-

Table 4.5: Table showing the results of the qualitative longevity study

From the table above and by viewing the corresponding pictures, the visual limit for the visual and Matlab processed images for Lumiscene and Bluestar Magnum appears less than the other reagents. Grodsky and Lumiscene Ultra have visual upper limits equal to each other. Contradictory to the graphs in figures 4.14 and

4.15, the upper limit for Grodsky is greater than that of Lumiscene Ultra when considering the images processed in Matlab. The ring effect described above is present earlier in the reaction for Lumiscene and Lumiscene Ultra than the other reagents. For the unaltered images, this ring forms at equal times for both Lumiscene Ultra and Grodsky. In the Matlab processed images, ring formation appears later in the Grodsky sample than that of Lumiscene Ultra. The Hemascein samples were visualised up to the mesured two and a half hours after the reaction which of course was a much longer time than the luminol based reagents. Also, there was no considerable ring formation.

Interestingly, the Hemascein images' level of light intensity is greatly increased in the 20 minute image and then there is a decrease in light intensity in the two and a half hour image. This is in accordance with the graph in figure 4.16. Also noticeable in the 20 minute image is the apparent increase in background intensity, especially along the left edge of the substrate in the image.

4.2.3 Reaction Endpoint Estimation

Based on the results obtained from both the quantitative and qualitative studies of the longevity of the reaction of each reagent with blood, a tentative limit of detection can be defined. The upper limit of detection was chosen at 10 AU and related to the amount of light emitted from a reaction which can reliably be seen with the unaided eye. The lower limit of detection was chosen at 5 AU and related to the amount of light emitted from a reaction which can reliably be visualised through computer enhancement. The figures listed below are for comparative purposes and are not absolute figures. Relative intensity is scaled in log units.
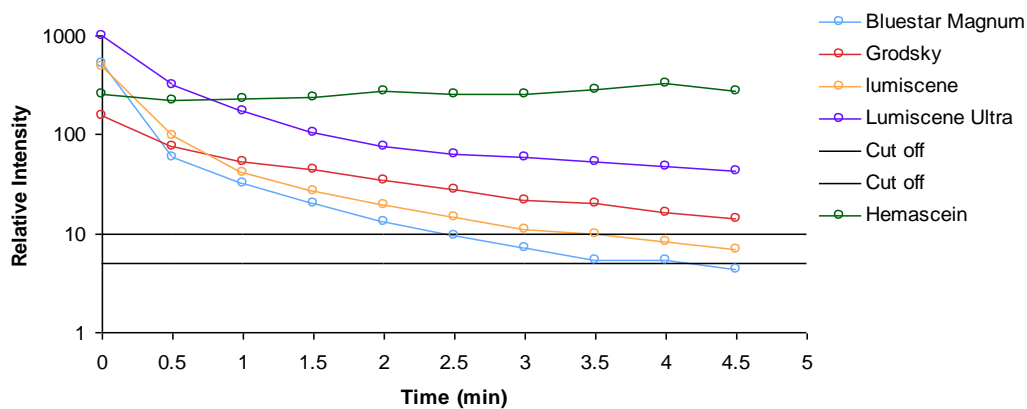
Figure 4.22: Graph showing the relative intensity vs. time and an estimation of the limit of detection for each of the reagents from 0 to 5 minutes post inception.
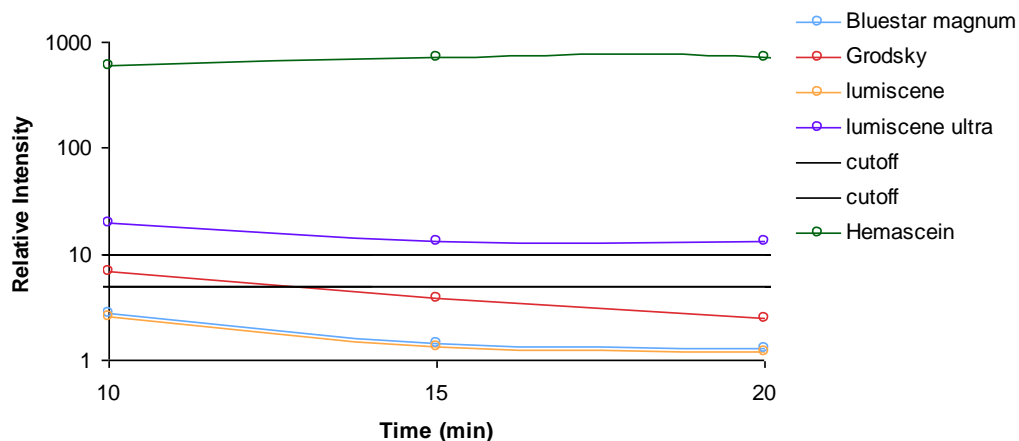
Figure 4.23: Graph showing the relative intensity vs. time and an estimation of the limit of detection for each of the reagents from 10 to 15 minutes post inception.

The following table contains the LOD values for each reagent based on the above graph. The values for the LODs were assigned by selecting the time interval value pertaining to the minimum concentration allowing successful detection of the positive reaction. Close calls, where the LOD line crossed the reagents line just before or just after a concentration value, were judged by whether the reaction could be seen in the images.

	Grodsky	Bluestar Magnum	Lumiscene	Lumiscene Ultra	Hemascein
LOD 10 AU	4.5-5	2-2.5	2.5-3	-	-

LOD 5 AU   10   4-4.5   4.5-5   -   -

Table 4.6: Shows the limit of detection for each of the reagents at 10 and 5 intensity units.

From the above table it can be seen that Bluestar Magnum has the shortest reaction length. Lumiscene had a slightly greater LOD than Bluestar Magnum at both 5 and 10 arbitrary units. Grodsky had a longer significantly longer reaction length than Bluestar Magnum and Lumiscene. Lumiscene Ultra, contrary to the images, appeared not to have a limit of detection as the intensity of the reaction at 20 minutes was greater than 10. Hemascein also had an intensity value greater than 10 after 20 minutes with an intensity value considerably greater than that of Lumiscene Ultra.

4.3 DNA and mRNA Analysis

4.3.1 DNA Quantitation

DNA Quantitation was important in this study, first to determine the amount of human DNA present to determine the amount of DNA solution that should be added to the PCR tube for STR amplification. The Identifiler™ PCR amplification kit requires a final input DNA concentration of 0.05-0.125ng/µl. If the input DNA is less or more than this range then STR profiling will be impaired (AmpFLSTR® Identifiler® User's Manual.

Also, DNA quantification is important to determine whether there are inhibitors present which could inhibit the PCR reaction. The Quantifiler™ kit contains an internal PCR control (IPC) sequence which is co-amplified with the sample. The IPC is a synthetic oligonucleotide sequence which produces fluorescence which is detected by the quantification system. The IPC is useful for determining whether the DNA present in the sample is unsuitable for amplification by PCR by predicting the presence of PCR inhibitors. If PCR inhibitors are present the IPC is either not amplified or is amplified with a high Ct value. If there is no human DNA present, or the DNA is degraded, the IPC is still amplified. (M. Barbisin et al Validation of a multiplexed system for quantification of Human DNA and human male DNA and detection of PCR inhibitors in biological samples (2007) Proceedings of the 18th

Promega Genetic Identity Conference + DNA IQ™ System-small sample casework protocol)

Results from the Quantifiler™ quantification for each of the samples were placed in an EXCEL spreadsheet. This included the amount of human DNA (ng) present in each sample and the Ct value for both the sample and the IPC. The Ct value is the cycle number at which detectable fluorescence passes the present threshold. (DNA analyst Training Laboratory Training Manual Quantifiler Quantitation procedure)

The results from the Ct output comparing human DNA and the IPC showed that there were no PCR inhibitors in any of the samples. All IPC Ct values were in accepted level of 27+-0.5.

Based on the amount of DNA obtained from the sample, the amount of DNA solution which should be added to the PCR tube was calculated. After the volume was established, the amount of DNA utilised from the sample for PCR amplification per micro litre of DNA solution was calculated. The amount of DNA for each sample at both dilutions (1:10 and 1:100) was plotted on the following graph. The amount of DNA is on the y-axis in ng/μl and each reagent type is plotted as x-axis categories. Error bars are one standard deviation either side of the mean.

DNA Quantification 1:10
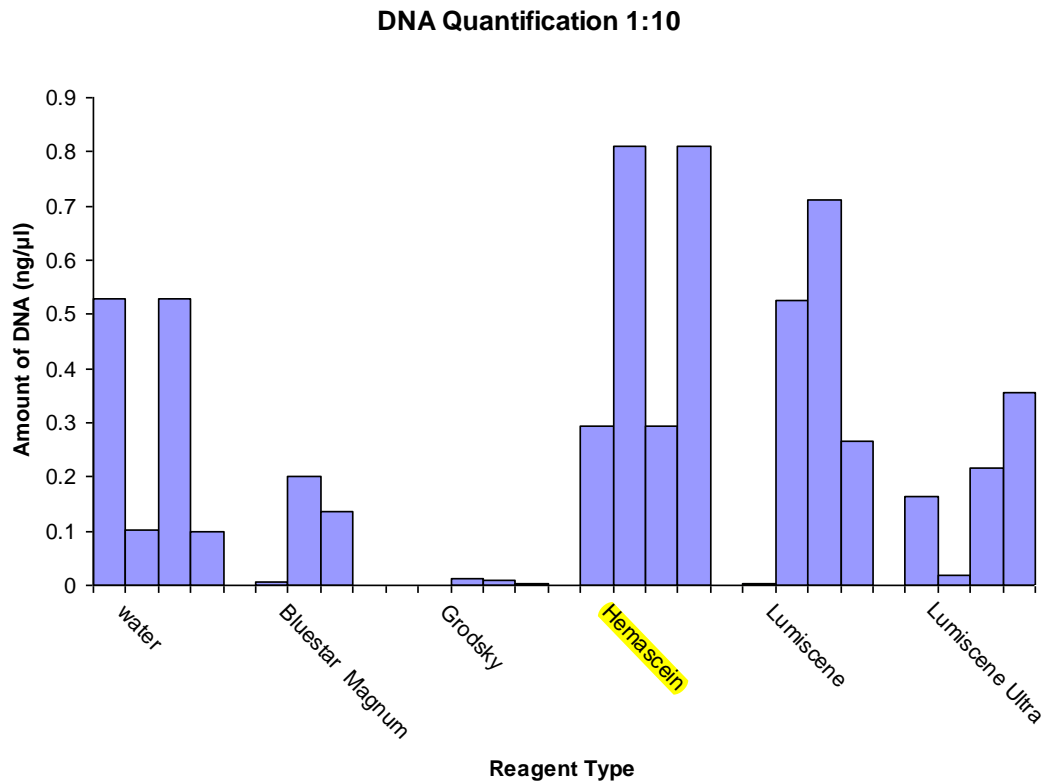
Figure 4.24 Graph of the results from quantification of the 1:10 samples

From the above graph it can be observed that all of the water, Hemascein, Lumiscene and Lumiscene Ultra treated quadruplicate samples were quantified. One sample from both Bluestar Magnum and Grodsky was undetected. The amount of DNA extracted from the Grodsky samples appears to be less than most of the other samples.
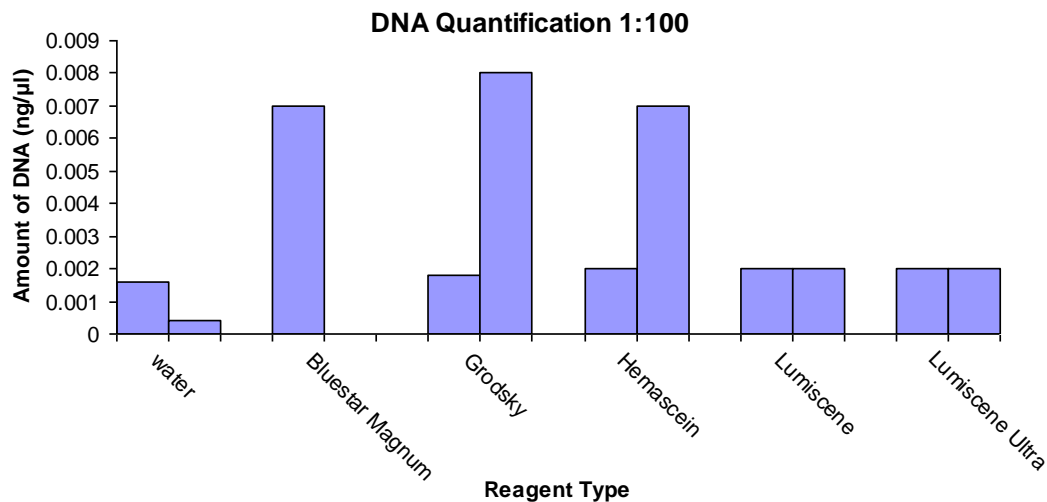
Figure 4.25: Graph of the results from quantification of the 1:100 samples

Most of the samples had very low amounts of DNA extracted from them. One sample each from the Bluestar Magnum, Grodsky and Hemascein sample duplicates gave higher amounts of DNA. One of the Bluestar Magnum samples was undetected.

STR Amplification

STR amplification was carried out to determine if any of the reagents interfere with DNA profiling. The Identifiler™ kit amplifies 15 tetranucleotide repeat loci and the gender determining marker, Amelogenin, in a short tandem repeat (STR) multiplex assay. After a PCR reaction of 28 cycles the data was analysed by the ABI Prism™ 330 DNA analyser and the STR profile assigned using Genotyper™ version 3.7. This gave the size of each STR and the height of each corresponding peak height measured in relative fluorescence units (RFU) at each allele. The allele name, peak height and size of the STRs for each sample were recorded in an EXCEL spreadsheet and graphs were plotted to visualise the data and determine the extent of degradation, if any, caused by each of the reagents.

The following graphs show the relationship between peak height and STR size for each of the profiles of the 1:10 diluted bloodstains treated by the various treatments. Each graph shows each data point from all profiles for each given treatment. Also included is the linear line equation in the form of y=mX +c where mX describes the slope of the line and c describes the y-intercept. The slope of the line (mX) can be used as an indication of DNA degradation since larger STRs are more susceptible to degradation than shorter STRs (Identifiler manual reference). The y-intersept (c) can be used as an approximation of the average peak height.

Grodsky 1:10

$y = -0.0257x + 152.46$

(y-axis: Peak Height (RFU), 0 to 500; x-axis: STR Size (BP), 0 to 400)
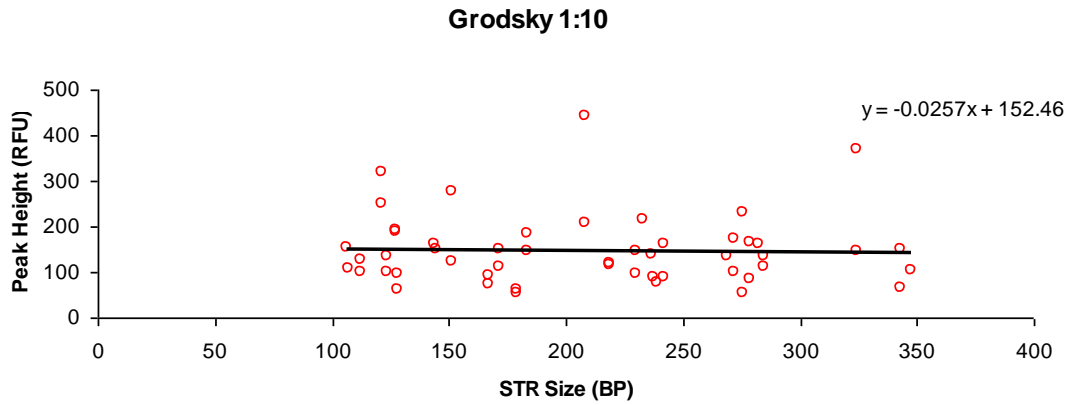
Figure 4.26 STR size vs. peak height for the Grodsky treated 1:10 bloodstains

Figure 4.27: STR size vs. peak height for the Bluestar Magnum treated 1:10 bloodstains

Lumiscene 1:10

$y = -0.0569x + 1069.7$

(y-axis: Peak Height (RFU), 0 to 4000; x-axis: STR Size (BP), 0 to 400)
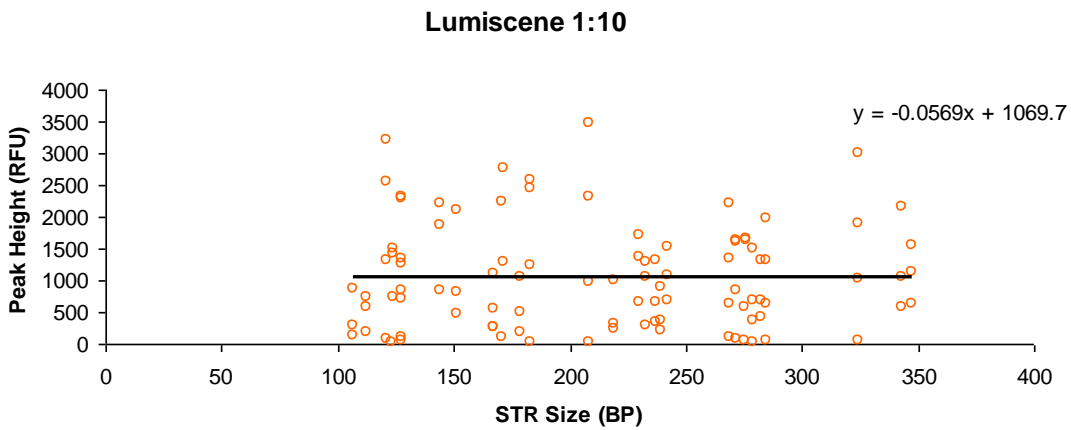
Figure 4.28: STR size vs. peak height for the Lumiscene treated 1:10 bloodstains

Lumiscene Ultra

$y = -0.9527x + 1017.5$

(y-axis: Peak Height (RFU), 0 to 3000; x-axis: STR Size (BP), 0 to 400)
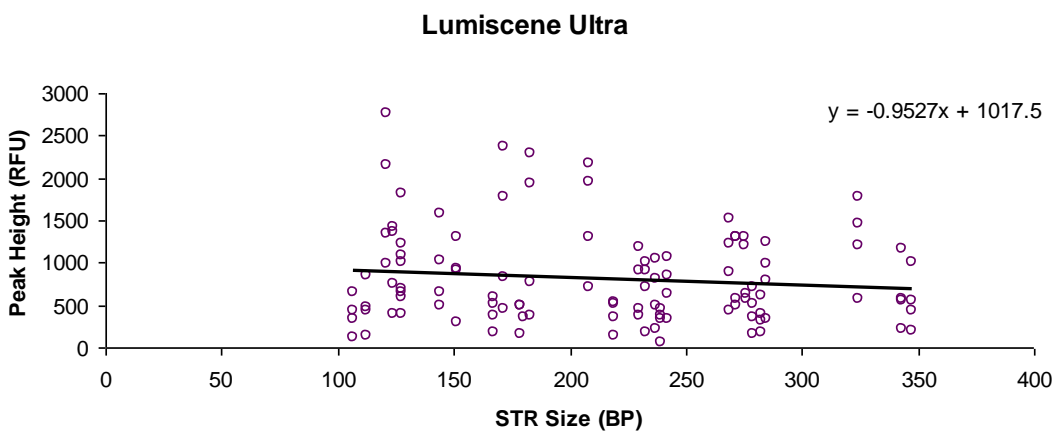
Figure 4.29 STR size vs. peak height for the Lumiscene Ultra treated 1:10 bloodstains

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Hemascein 1:10

$y = 0.9073x + 1615.2$

(Y-axis: Peak Height (RFU); X-axis: STR Size (BP))
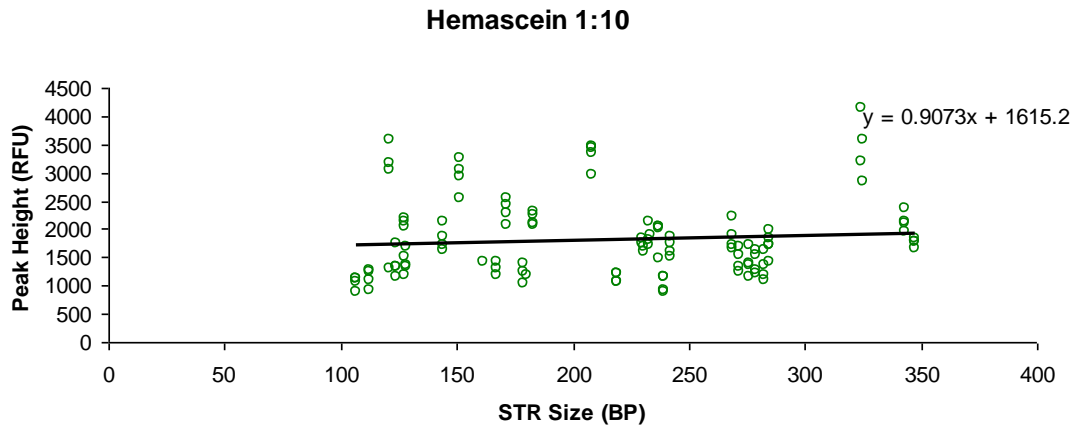
Figure 4.30 STR size vs. peak height for the Hemascein treated 1:10 bloodstains

Water 1:10

$y = 3.8585x + 1933.4$

(Y-axis: Peak Height (RFU); X-axis: STR Size (BP))
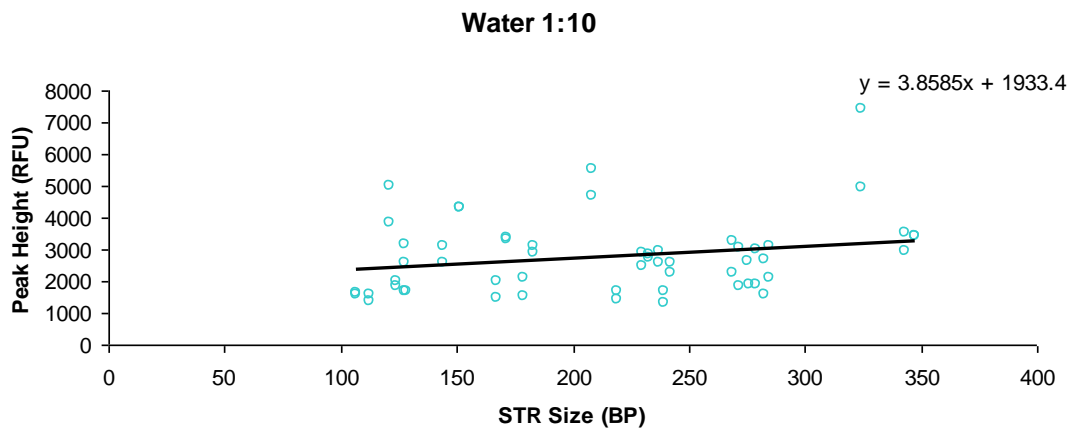
Figure 4.31 STR size vs. peak height for the Water treated 1:10 bloodstains

From the above graphs it can be observed that the water treated 1:10 bloodstains show the least degradation due to the positive slope on the line equation. There appears to be more STRs with longer bp repeat sequences than the other treatments. Also apparent is the over all peak height, as per the y-intercept, is greater than the other reagents meaning an overall stronger fluorescent signal is displayed by the water treated samples. The Hemascein graph also shows a positive slope on the line equation, however, the slope is less than that of the water graph. The Lumiscene and Grodsky line equations show an almost straight line while the Lumiscene Ultra and Bluestar Magnum line equations show a definite negative slope. The Grodsky line equation has the lowest value for the y-intercept followed by Lumiscene Ultra, Lumiscene, Hemascein, Bluestar and water treated samples, respectively.

The following graph shows the average peak height of each STR measured in relative fluorescent units (y-axis) at a given allele (x-axis) for each of the treated 1:10 samples. Profiles where the total DNA content was 0.1ng/µl or less were excluded from the graph. At this value the profiles appear to be severely incomplete or non-existent. All of the Godsky treated samples failed to generate DNA amounts over 0.1ng/µl. However, because profiles were still obtained from these samples, they were plotted on the following graph. The alleles are arranged along the x-axis in terms of the size of their relative STR. Alleles on the left hand side of the graph contain STRs which are shorter than the alleles on the right hand side of the graph. The X and Y alleles refer to the Amelogenin sex determining STRs for those alleles.
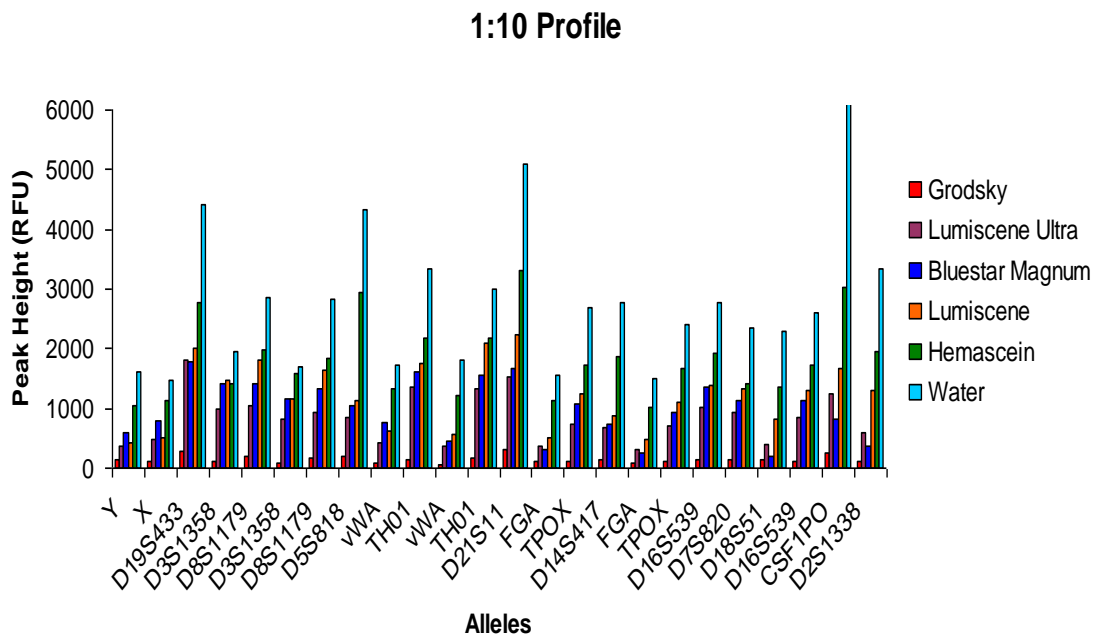
Figure 4.32: Average peak height for each allele

The average peak height for the water treated samples appears greater than the other samples over all the alleles. The average peak heights for the Hemascein treated samples are the next highest. The Lumiscene and Bluestar Magnum peak heights are relatively similar apart from the alleles pertaining to longer STRs. Grodsky has very low peak height values however the DNA content in these samples was less than the other treatments as shown in figures 4.24 and 4.32.

Multiple regression on the line slope for each of the repeats and for each reagent was undertaken to statistically show whether one reagent out performs the other in terms of degradation. Examination of the figures shows that the amount of DNA available for PCR has a significant effect on the peak height of the STR profiles. To eliminate this effect of DNA concentration each reagent was tested against each other using multiple regression. Below is a table giving the results.

	Bluestar Magnum	Hemascein	Lumiscene	Lumiscene Ultra	Water
Grodsky	0.928	0.457	0.467	0.831	0.0946
Bluestar Magnum		0.115	0.125	0.0796	0.0547
Hemascein			0.177	0.0484	0.146
Lumiscene				0.0678	0.0710
Lumiscene Ultra					0.0930

Table 4.7: Table of p-values from the multiple regression

The treatments could not be separated significantly based on the p-values obtained in the above table except for Hemascein outperforming Lumiscene Ultra. The average slope value was greater in the water treated samples than any of the other treatments followed by Hemascein, Lumiscene, Bluestar, Grodsky and Lumiscene Ultra respectively.

Multiple regression on the peak height for each of the STRs and for each reagent was then undertaken to statistically show whether one reagent out performs the other in terms of degradation. Examination of the figures shows that the amount of DNA available for PCR has a significant effect on the peak height of the STR profiles. To eliminate this effect of DNA concentration each reagent was tested against each other using multiple regression. Below is a table giving the results.

	Bluestar Magnum	Hemascein	Lumiscene	Lumiscene Ultra	Water
Grodsky	2.22E-6	6.83E-12	1.81E-8	0.000388	2.14E-14
Bluestar Magnum		5.5E-5	0.900	0.000325	0.000675
Hemascein			8.53E-7	4.81E-12	0.00301
Lumiscene				0.0493	2.13E-6
Lumiscene					5.63E-7

Ultra

Table 4.8: Table of p-values from the multiple regression

The *p*-values show that the water treatment performed significantly better than all of the other treatments. Lumiscene Ultra, Bluestar Magnum, Hemascein and Lumiscene performed significantly better than Grodsky. Hemascein also performed significantly better than Bluestar Magnum, Lumiscene and Lumiscene Ultra. Bluestar Magnum and Lumiscene performed significantly better than Lumiscene Ultra, though they could not be significantly separated and thus performed similarly.

The 1:100 profiles were then analysed. The following graphs show the relationship between peak height and STR size for each of the profiles of the 1:100 diluted bloodstains treated by the various treatments. Each graph shows each data point from all profiles for each given treatment. The line equation was excluded form these graphs as these samples were only run in duplicate (except for the Bluestar Magnum and Lumiscene Ultra treatments where only one sample produced a profile) and therefore there was deemed insufficient data to be described by the line equation.

1:100 Grodsky
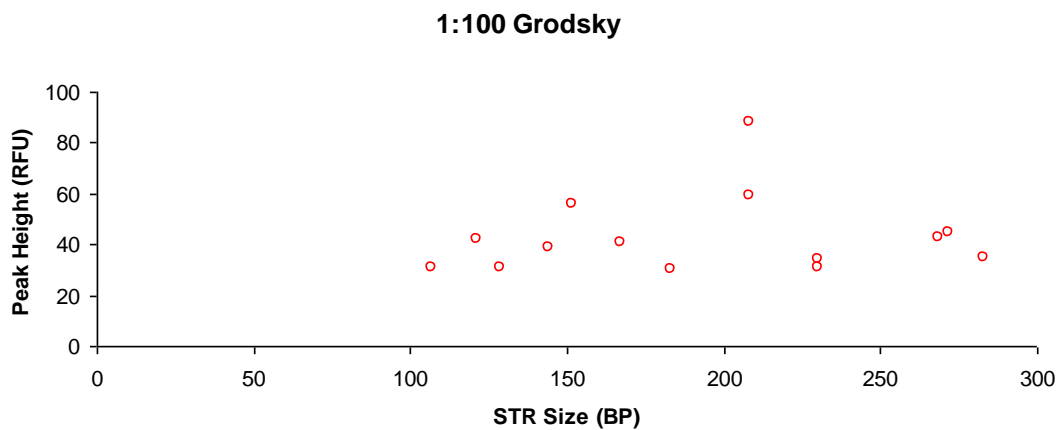
Figure 4.33: Grodsky peak height vs STR size

1:100 Bluestar Magnum
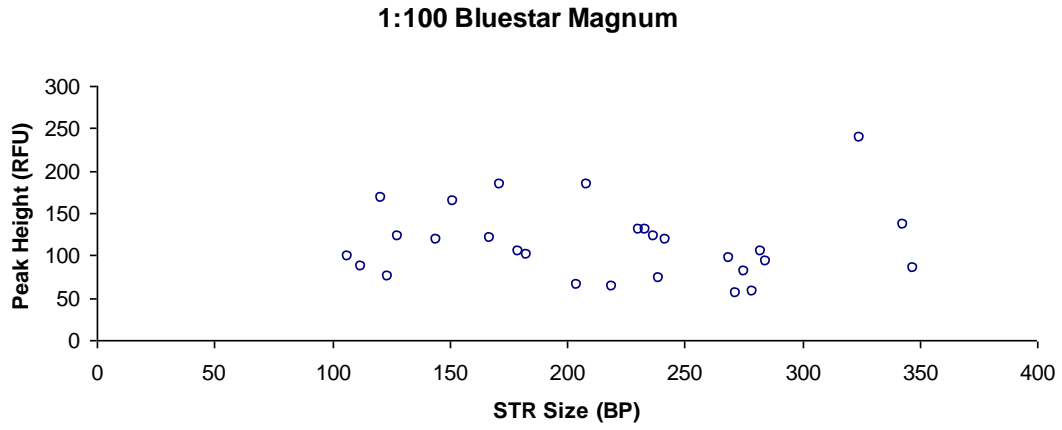
Figure 4.34: Bluestar peak height vs STR size
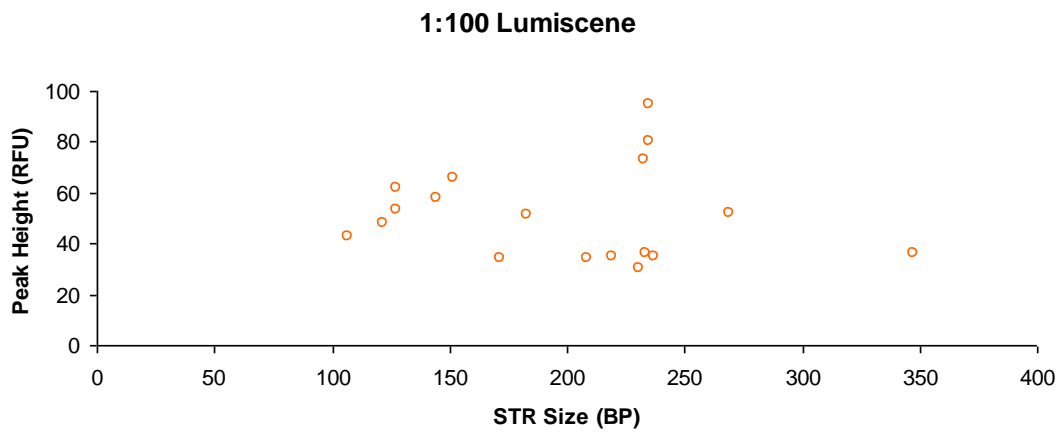
1:100 Lumiscene

Figure 4.35: Lumiscene peak height vs STR size

1:100 Lumiscene Ultra
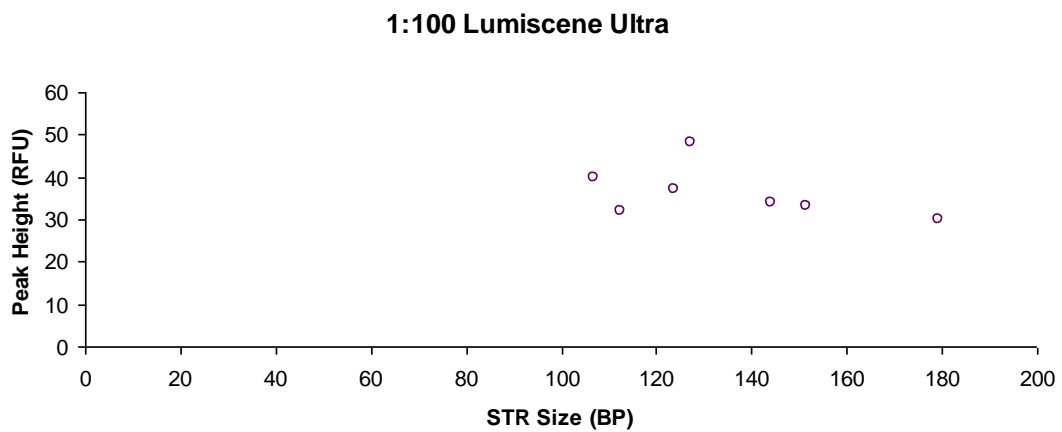
Figure 4.36: Lumiscene Ultra peak height vs STR size

1:100 Water
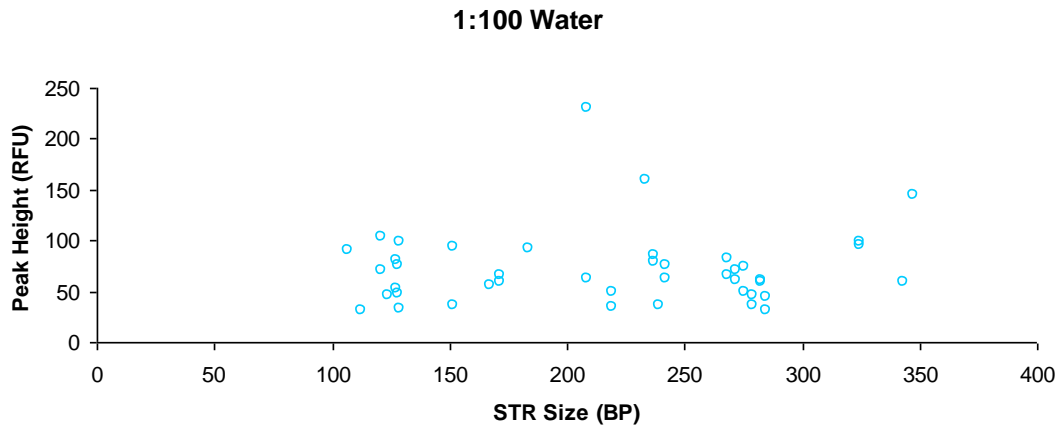
Figure 4.37: Water peak height vs STR size
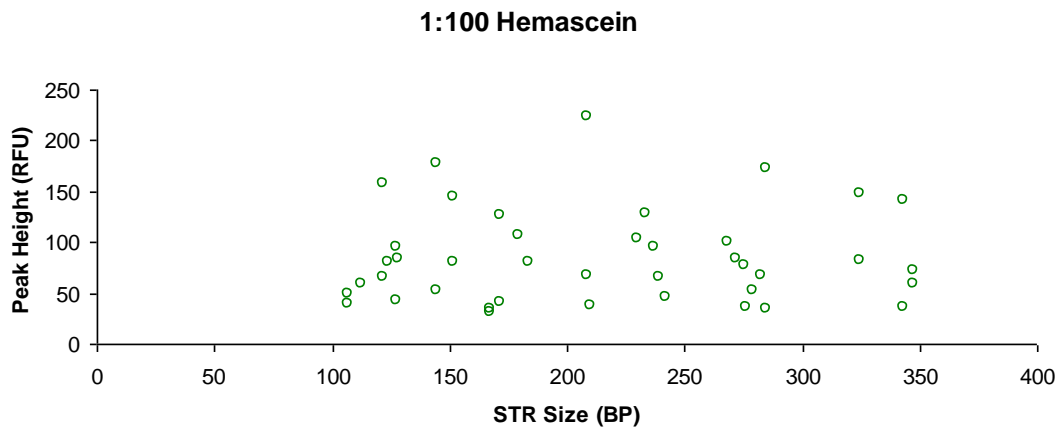
1:100 Hemascein

Figure 4.38: Hemascein peak height vs STR size

From the above graphs, the water, Hemascein and Bluestar Magnum samples appear to have the highest peak height values. Grodsky, Lumiscene and Lumiscene Ultra all appear to peak height values lower than the aforementioned treatments. Also apparent from these graphs is the apparent loss of longer STRs from those treatments which produced lower peak heights including Grodsky, Lumiscene and Lumiscene Ultra.

The following graph shows the average peak height of each STR measured in relative fluorescent units (y-axis) at a given allele (x-axis) for each of the treated 1:100 samples. All of the samples failed to generate DNA amounts over 0.1ng/µl.

Therefore all peak heights were plotted regardless of the completeness of the profile. The alleles are arranged along the x-axis in terms of the size of their relative STR. Alleles on the left hand side of the graph contain STRs which are shorter than the alleles on the right hand side of the graph. The X and Y alleles refer to the Amelogenin sex determining STRs for those alleles.
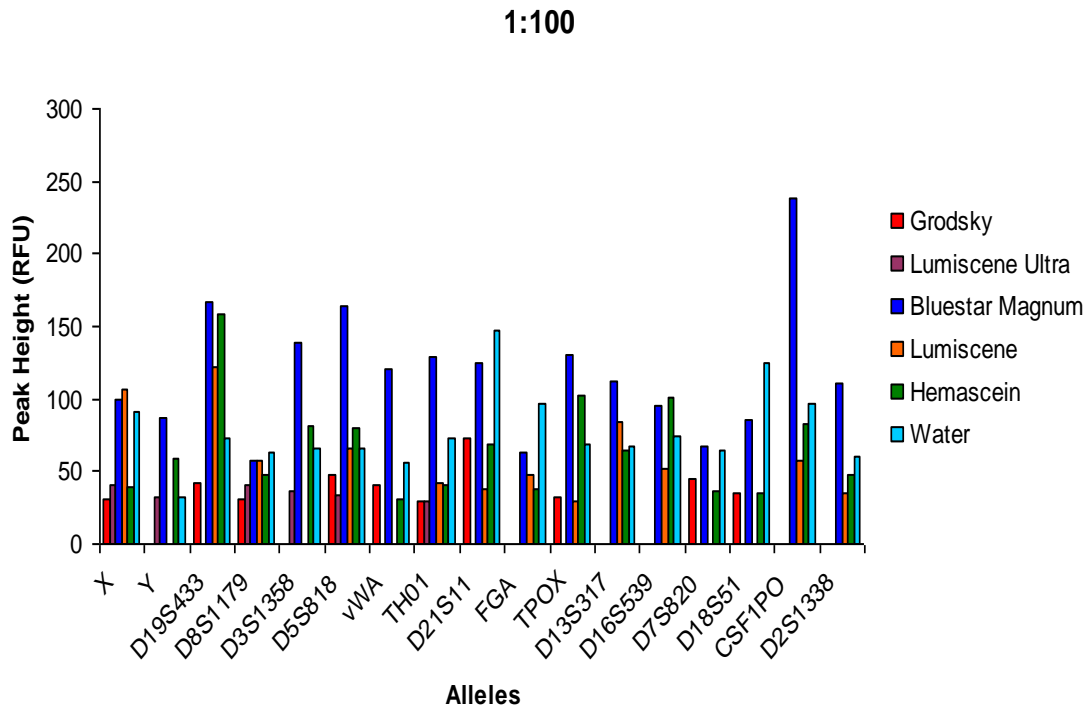
1:100

Figure 4.39 Graph showing the average peak height for each allele for the different treatments of the 1:100 diluted bloodstain samples.

The average peak height for the Bluestar Magnum treated samples appears on average to be greater than the other samples, which is contradictory to the results from the 1:10 diluted samples. The Hemscein and water peak height values appear to be higher than that of Lumiscene, Lumiscene Ultra and Grodsky. The later mentioned treatments seem quite similar in terms of peak height. However, dropout is observed at many of the alleles, particularly with the Lumiscene Ultra treated samples.

Multiple regression on the peak height for each of the repeats and for each reagent was then undertaken to statistically show whether one reagent out performs the other in terms of degradation. Examination of the figures shows that the amount of

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DNA available for PCR has a significant effect on the peak height of the STR profiles. To eliminate this effect of DNA concentration each reagent was tested against each other using multiple regression. Below is a table giving the results.

1:100

	Lumiscene	Lumiscene Ultra	Hemascein	Grodsky	Water
Bluestar Magnum	4.85E-7	4.29E-5	2.04E-5	0.000829	0.0152
Lumiscene		0.0490	2E-5	0.255	0.925
Lumiscene Ultra			0.000199	0.312	0.401
Hemascein				0.000707	0.000113
Grodsky					0.0995

Table 4.9: Table of p-values from the multiple regression

The p-values show that the Bluestar magnum treatment performed significantly better than all of the other treatments. Hemascein performed significantly better than the other treatments apart from Bluestar Magnum. Lumiscene performed significantly better than Lumiscene Ultra but not the Grodsky or water treatments. However, the average peak intensity value was higher for Lumiscene. The water treatment could not be significantly separated from Grodsky or Lumiscene Ultra though had higher average peak height values. Grodsky performed better than Lumiscene Ultra, though not significantly.

The profiling success of each treatment was then evaluated by assessing the average percentage of alleles which could be successfully typed in both the 1:10 and 1:100 bloodstain samples. For the 1:10 bloodstains, only samples with a final PCR DNA volume greater than 0.1ng/µl were considered. For the 1:100 diluted bloodstains all peak heights from all profiles were considered except those which were labelled "undetected" in the quantitation. The following graph shows the percentage of alleles successfully typed (y-axis) with the different treatments (x-axis). Error bars are one standard deviation away from the mean.
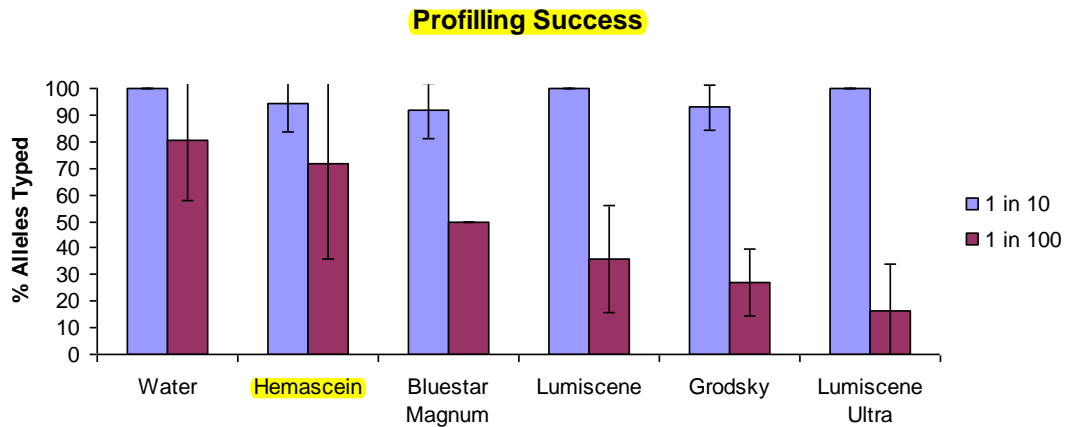
Figure 4.40: Bar chart showing the relative DNA profiling success from bloodstains treated with the various reagents

From the above graph, the percentage of alleles typed at the dilution 1:10 were close to 100% meaning full or nearly full profiles were obtained. The water, Lumiscene Ultra and Lumiscene samples all produced full profiles. The Hemascein, Bluestar Magnum and Grodsky treated average percentage values were 94.6%, 91.6% and 92.8%, respectively.

For the 1:100 diluted bloodstains, the profiling success was reduced in all of the treatments compared to the 1:10 bloodstain samples. The average percentage value for the water samples was greater than the other treatments at 80.4%. Hemascein on average successfully typed over half of the alleles, 71.4%. Bluestar Magnum successfully typed exactly half the alleles while Lumiscene, Grodksy and Lumiscene Ultra successfully typed less than half the total alleles with percentages 35.7%, 26.8% and 16.1%, respectively.

4.4 Application Method

4.4.1 Droplet Size and Density

The first experiment was to evaluate the spray droplet size and spray density of each sprayer. The Images were first processed in Photoshop to achieve the best contrast between background and the phenolphthalein droplets to enable easy

counting of the 1mm² squares covered by each droplet. Images from this experiment are shown below and are of the most representative part of the sprayed sheet of graph paper. The thicker lines represent 1cm² squares, each containing four 5mm² squares (minor grid lines) and each 5mm² square containing 25 1mm² squares (thinnest gridlines). This gives a total of 100 1mm² squares in each 1cm² area.
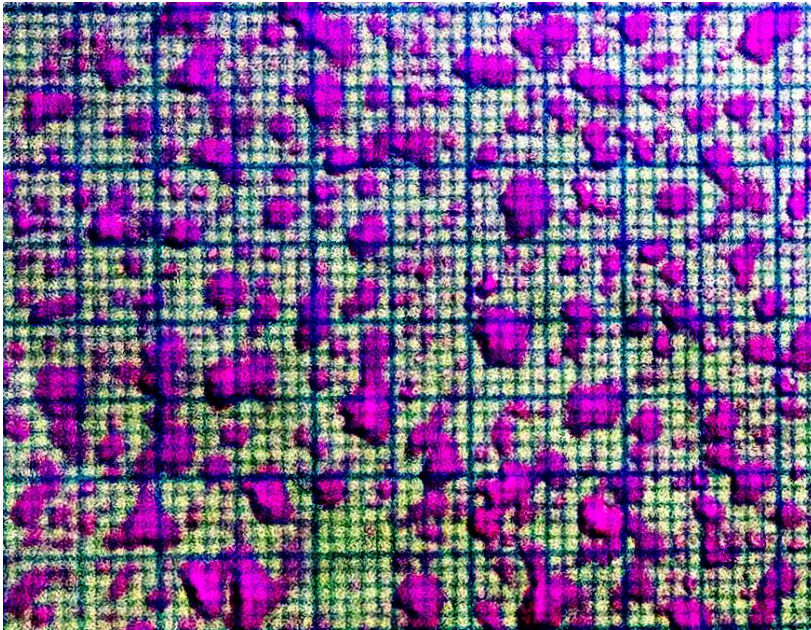
Hand Pump Sprayer
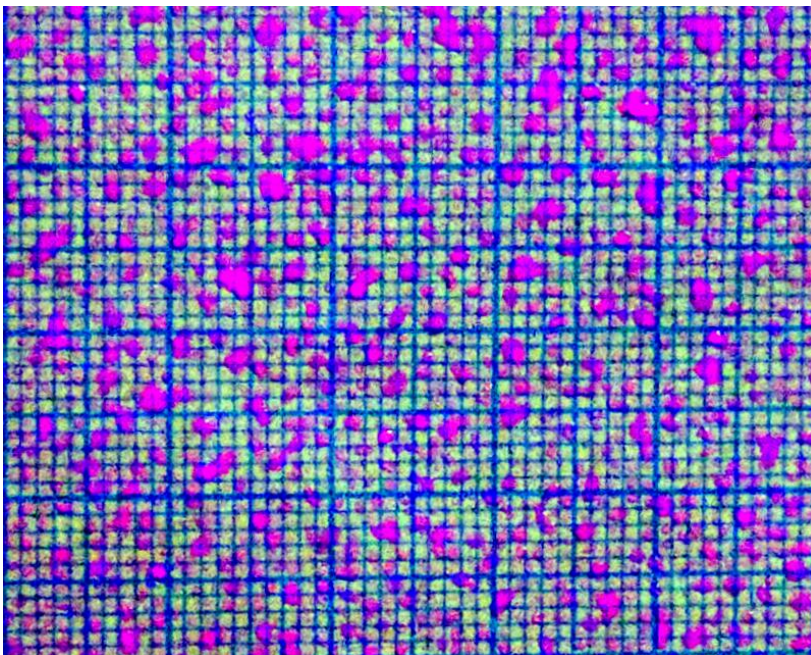
ABA Sprayer
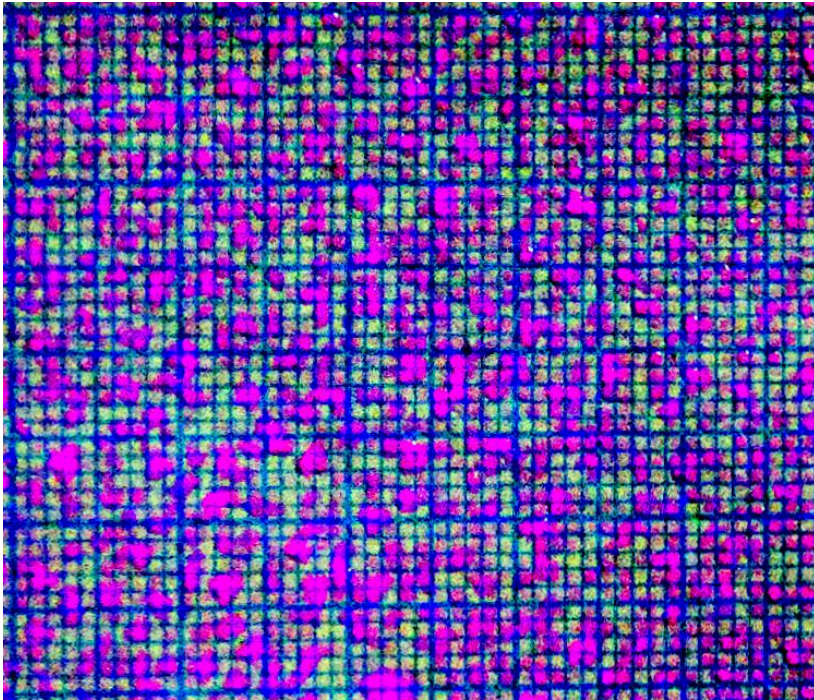
ABA Finger Sprayer
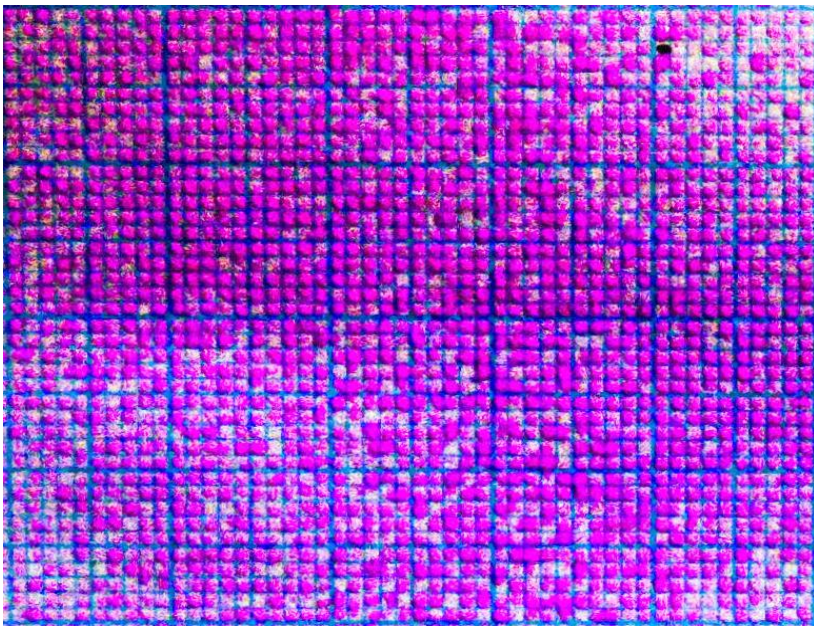
ECO spray
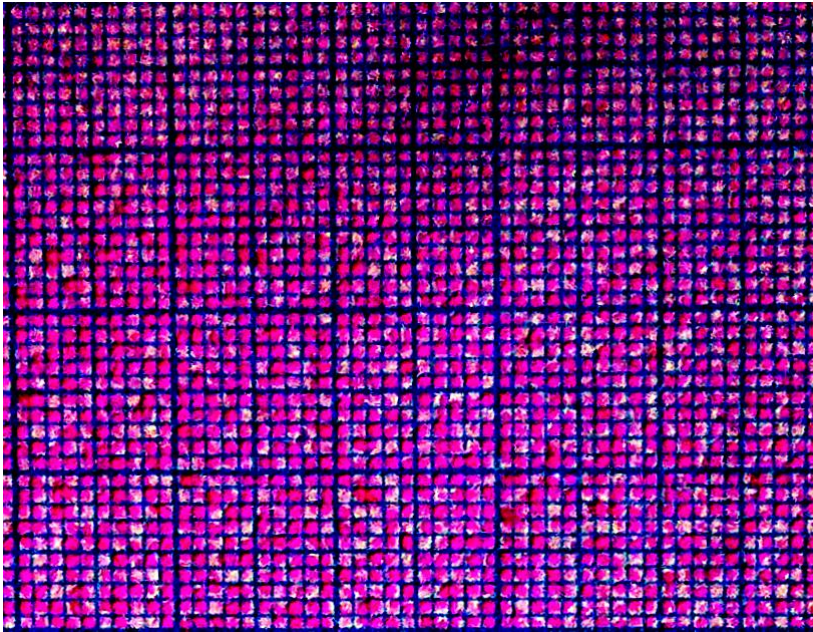
Spray Gun

Figure 4.41: Images showing the area covered and droplet size for each of the spray types

The above images show that the pump sprayer appears to have the largest droplets and the least coverage over the shown area with reference to how many of the 1mm² squares are covered. The ABA spray and the ABA finger spray appear to have similar droplet sizes. The density of the spray droplets appear to be slightly higher in the ABA finger spray sprayed samples rather than the ABA spray. The ECO spray and the spray gun have very similar droplet sizes and density. The sizes of the phenolphthalein droplets produced by both of these sprays seem confined to filling individual 1mm² squares. The droplet size for the ECO spray and the spray gun was therefore considered to be 1mm². Also observed from the ECO spray and spray gun images is the amount of coverage by these two sprays. A greater number of the 1mm² squares are more evenly and thoroughly covered by these two sprayers than the other application methods.

Values of droplet size and amount of solution coverage were based on the number of 1mm² squares covered with the phenolphthalein solution. The number of droplets in a given 1cm² area were then counted. These values were recorded in EXCEL and graphs were created for visualisation of the data.

Below is a scatter plot showing the relationship between the number of droplets present on each of the selected 1cm² squares vs. the size of those droplets. Droplet

size was measured by counting the number of 1mm² squares on the graph paper covered by each droplet. The number of droplets per selected 1cm² square is on the x axis.
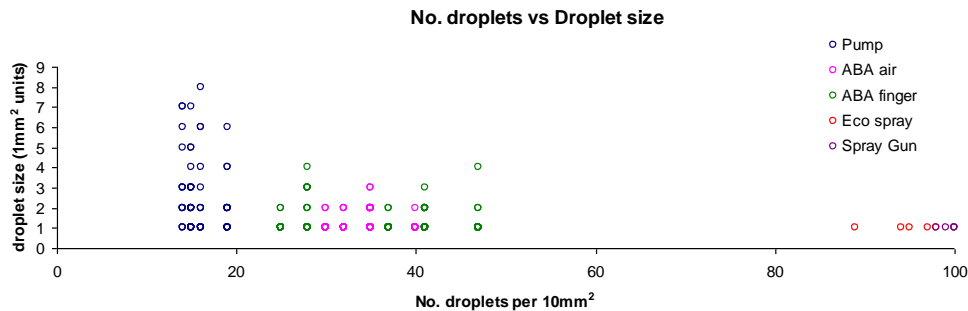
Figure 4.42: Graph showing the droplet size vs number of droplets for each of the different spray

Immediately apparent from this graph is the vast difference between the pump sprayer, the ABA spray and the ABA finger spray to the ECO spray and the spray gun. The droplets produced by ECO spray and the spray gun appear to have more smaller sized droplets than the other sprayers. ABA air and ABA finger appear to have a similar relationship between droplets size and number of droplets. However, the data spread for ABA finger spray for the number of droplets per 1cm² area appears larger. The pump sprayer had some droplets which were a lot larger than those produced by the other sprayers and had fewer droplets per 10mm² area.

The next graph depicts the average droplet size based on how many 1mm² squares each single droplet covered within the selected areas. Error bars are shown as one standard deviation either way from the mean.
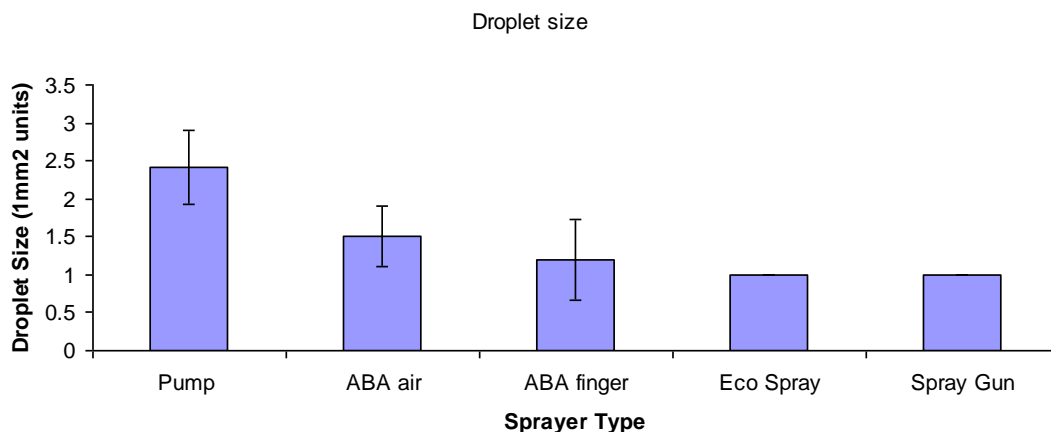
Figure 4.43: Graph showing the average droplet size for each of the different sprayer types

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In this graph, Eco spray and the spray gun have equal droplet size and the smallest average droplet size of all the participating sprayers. ABA finger had the next smallest mean followed by ABA air and lastly, the pump sprayer. In comparison to the previous graph, the average size for each sprayer was relatively small. As observed in the previous graph, some of the pump sprayer droplets were as large as eight 1mm² units. However, form this graph it can be observed that the majority of the droplets were smaller in size.

The question of interest is whether if the size of the droplets expelled from each sprayer is significantly smaller than droplets from the other sprayers. Multiple regression was performed to investigate this, taking into account also the number of droplets sprayed in a 10mm2 area. The results from this are in the table below.

	Aba spray	Aba finger	Eco spray	Spray gun
Pump	2.01E-15	1.88E-15	2.13E-53	2.21E-56
Aba spray		0.405	4.65E-16	6.55E-17
Abafinger			1.72E-14	2.99E-15
Eco-spray				0.975

Table 4.10: The *p*-values for the multiple regression testing each spray type against the others.

From these results it can be easily seen that the ECO spray and the spray gun have much smaller droplets for the number of droplets sprayed. However, ECO spray and the spray gun had a similar performance and are not significantly different from each other. ABA spray and ABA finger are significantly different from the pump sprayer, having smaller droplets for the number of droplets sprayed but are not significantly different from each other. From the average values based on the t-test, the spray gun has slightly smaller droplets for the number of droplets sprayed than that of Eco spray and ABA finger has smaller droplets than ABA spray, though not significantly.

The next question of interest was whether a spray covered more area than another spray, regardless of droplet size. The graph below shows the results from this experiment. Along the x axis are the types of sprayers utilised in this experiment and on the y axis is the percentage of 1mm² squares covered in the 10mm² selected area. Plotted on the graph is the average percent of 1mm² covered and not covered

for each spray type in five selected 10mm² squares. The error bars included in the graph are one standard deviation either way of the mean.
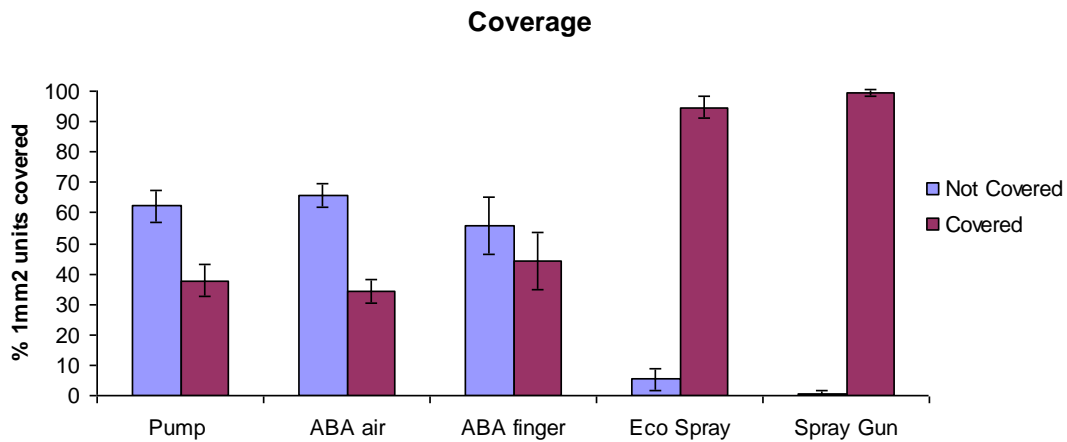
Coverage

Figure 4.44: Graph showing the area covered and not covered by the spray of each of the different spray types

Immediately apparent is the much greater extent of coverage of the ECO spray and the spray gun compared to the other spray types. The ECO spray and the spray gun have similar results. The other sprays, the pump sprayer, ABA spray and the ABA finger spray, have roughly equal coverage and non coverage.

Regression was used to determine whether there is a significant difference between the sprays in terms of coverage. The results of this are shown in the table below.

	Aba spray	Aba finger	Eco spray	Spray gun
Pump	0.257	0.739	3.95E-8	5.46E-9
Aba spray		0.311	5.95E-9	3.98E-10
Abafinger			3.68E-6	1.33E-6
Eco-spray				0.0180

Table 4.11: The *p*-values for the multiple regression testing each spray type against the others.

The spray gun significantly covered more 1mm² units than all the other spray types. The Eco spray significantly covered more 1mm² units than the ABA spray, the ABA finger spray and the pump spray. ABA finger covered more 1mm² units than ABA spray and the pump sprayer but not significantly and the pump sprayer covered more area than ABA spray, though also not significantly.

4.5 Morphoanalytical Study- Reagent Type

This first set of morphoanalytical studies sought to act as a baseline for the subsequent morphoanalytical studies, the application methods and fixative/shear thinning agent experiments. Only the vertical non-porous surface was rather than the horizontal-porous surface as well because it was found experimentally that the extent of reagent "running" and "pooling" was very great.

4.5.1 Vertical Non-porous Surface

The results on how the ability of the different reagent types to prevent blood pattern destruction on vertical non-porous surfaces are displayed in the table below. The different reagents were scored by the following criteria: If most of the lines of a particular sample could not be resolved then the letter N was assigned. If most of the lines could be resolved, then the letter Y was assigned. The letter r was assigned if the image showed signs of luminol running down away from the lines.

	3mm			2mm			1mm		
	Tile 1	Tile2	Vinyl	Tile 1	Tile 2	Vinyl	Tile 1	Tile 2	Vinyl
Grodsky	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr
Bluestar	Nr	Yr	Nr	Yr	Nr	Yr	Nr	Nr	Nr
Lumiscene	Yr	Nr	Nr	Nr	Yr	Nr	Nr	Nr	Nr
Lumiscene Ultra	Yr	Yr	Yr	Yr	Yr	Nr	Nr	Nr	Nr
Hemascein	Y*	Y*	Y*	Y*	Y*	Y*	Y*	N	Y*

N= cannot distinguish lines
Y= can distinguish lines
r = "running"

Table 4.12: Results of the effect of the different reagents on blood patterns on the two different substrates

From the above table it can be seen that Grodsky luminol, when sprayed with the air gun for three seconds, causes running when sprayed on bloody lines and that the lines can not be resolved. This result was similarly found with Bluestar Magnum, Lumiscene. Lumiscene Ultra managed to resolve lines down to 2mm but failed to

resolve the 1mm lines. <mark>The Hemascein treated bloodstains managed to resolve bloody lines for most of the samples</mark> except one of the 1mm samples. If the photographs of the hemascein reaction were taken within 30 seconds then the lines were well defined. Otherwise, the lines became blurred together though did not "run" as with luminol. However, the hemascein used in this experiment was combined with ABA fix, as instructed by the manufacturer's recommendation. The following pictures represent some of the samples described in the above table.
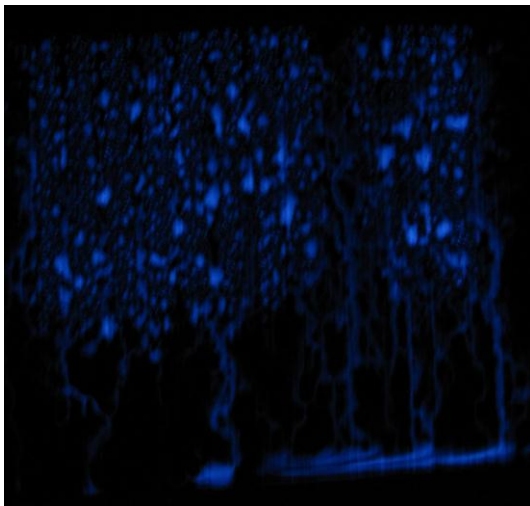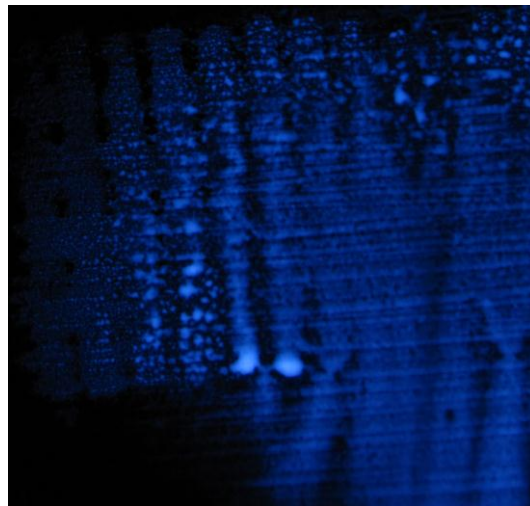
Grodsky- 3mm   Bluestar Magnum 3mm
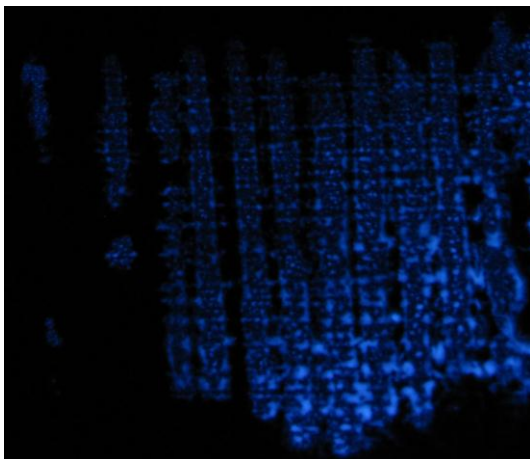
Lumiscene Ultra 2mm   Lumiscene 2mm
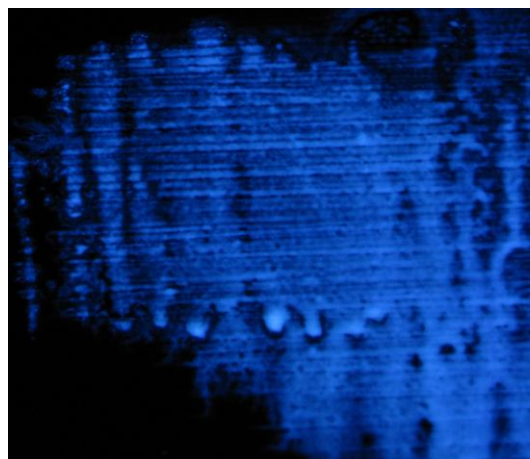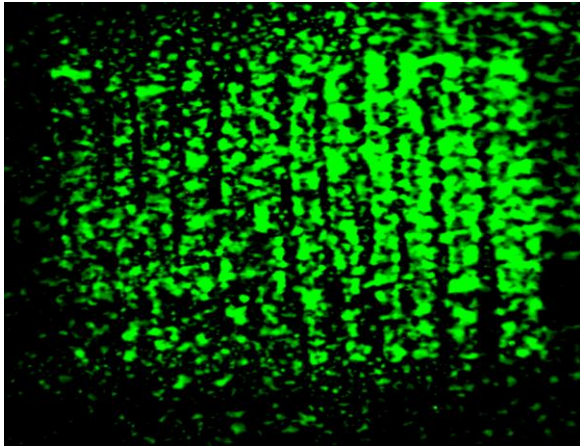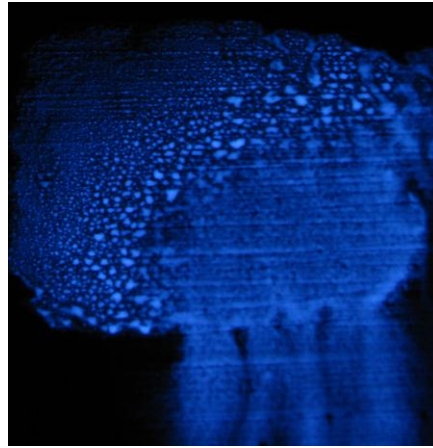
Hemascein 2mm   LumisceneUltra1mm

Figure 4.45: Images showing the ability of each of the reagents to reduce blood pattern distortion on vertical surfaces

4.6 Morphoanalitical Study- Application Method

This next section of experimental results sought to evaluate how each sprayer, when utilised with Grodsky luminol, was able to retain a blood pattern. That is, to investigate which spray type is best for preventing luminol destruction to blood patterns on vertical and horizontal non-porous surfaces.

4.6.1 Vertical Non-porous Surface

The results below display the ability of the different spray types to prevent blood pattern destruction on vertical non-porous surfaces. The different fixatives were scored by the following criteria: If most of the lines of a particular sample could not be resolved, then the letter N was assigned. If most of the lines could be resolved, then the letter Y was assigned. The letter r was assigned if the image showed signs of luminol running down away from the lines.

	3mm			2mm			1mm		
	Tile 1	Tile 2	Vinyl	Tile 1	Tile 2	Vinyl	Tile 1	Tile 2	Vinyl
Hand Pump	N r	N r	N r	N r	N r	N r	N r	N r	N r
Air	Nr	Yr	Yr	Y r	N r	N r	N r	N	N

pump

Finger pump	Yr	Yr	Yr	N r	Y r	Nr	N	N r	N r
Micro-sprayer	Yr	Yr	Yr	Y r	Yr	Y r	Nr	Nr	Nr
Air gun	Y	Y	Y	Yr	Y	Yr	Yr	Yr	Yr

Key:
N= cannot distinguish lines
Y= can distinguish lines
r = "running"

Table 4.13: Results of the effect of the different spray types on blood patterns on the two different substrates

From the above table it can be seen that luminol sprayed with the hand pump sprayer causes running when sprayed on bloody lines and that the lines can not be resolved. An example of the illegibility of 3mm blood lines when the pump sprayer is used to spray luminol is shown in figure 4.46. The ABA spray proved effective at resolving two of the three 3mm lines and one of the 2mm lines. When comparing the ABA spray with the pump spray, it can be observed that the ABA spray causes less running. This is shown in figure 4.46. The ABA finger spray managed to resolve each of the 3mm lines but failed to resolve all but one of the 2mm line samples. Running of the luminol reagent could still be observed as demonstrated in figure 4.46. The ECO spray managed to resolve all the 3mm and 2mm lines but failed to resolve the 1mm lines. In all cases running was observed. Figure 4.46 demonstrates this. The spray gun was the best at resolving blood lines with resolution down to the 1mm lines. The spray gun also prevented running in samples pertaining to the 3mm and 2mm lines. However, running was still observed in the 1mm samples. Figure 4.46 shows the 3mm resolved lines with no running effect while figure 4.46 shows most of the 1mm lines are resolved although running is apparent.
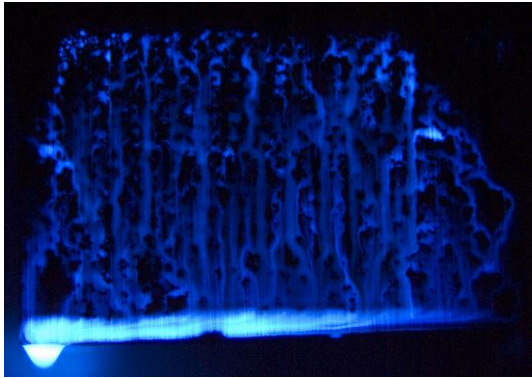
3mm Spray Pump
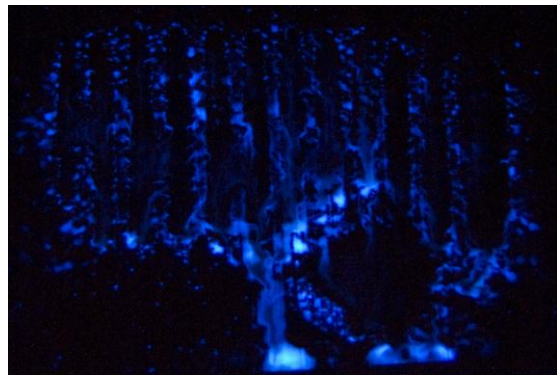
3mm ABA spray
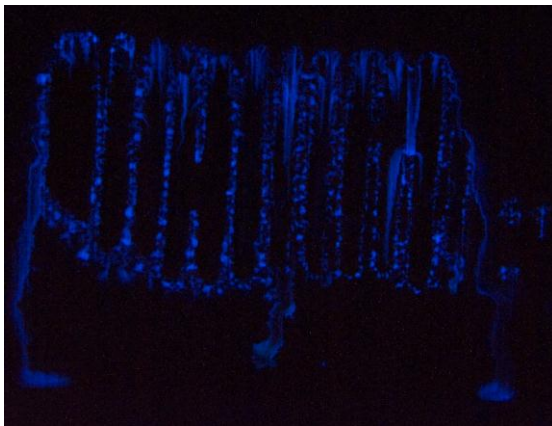
3mm ABA Finger

3mm ECO spray
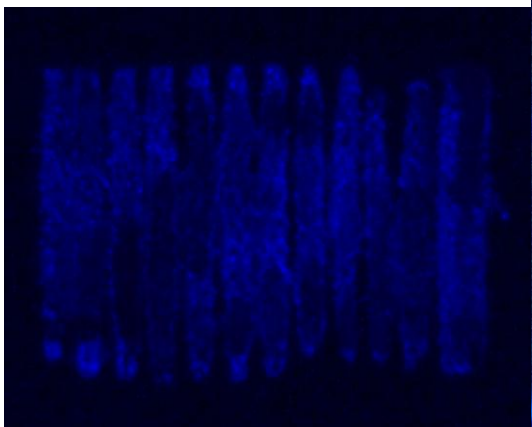
3mm Spray Gun

1mm Spray Gun

Figure 4.46: Images showing the ability of each of the different spray types to reduce blood pattern distortion on vertical surfaces

4.6.2 Horizontal Non-porous Surface

The results displaying the ability of the different spray types to prevent blood pattern destruction on horizontal non-porous surfaces are shown in the table below. The values obtained from measuring each blood line and space before treatment and the same lines and spaces after luminol treatment were recorded onto an EXCEL spreadsheet. The difference between these two measurements was then obtained to evaluate the extent of distortion occurring to the lines after luminol treatment. Multiple regression was then undertaken to statistically show whether one spray type out performs the other based on the difference values. Below is a table giving the results of the 3mm line samples.

3mm								
	Eco Spray		Aba Spray		Aba finger		Sprayer	
	Lines	Spaces	Lines	Spaces	Lines	Spaces	Lines	Spaces
Air Gun	0.929	0.824	0.308	0.451	0.0755	0.0150	-	-
Eco Spray			0.366	0.346	0.0899	0.0234	-	-
Aba Spray					0.135	0.189	-	-
Aba Finger							-	-

Table 4.14: The *p*-values for the multiple regression testing each spray type against the others.

The pump sprayer failed to produce any readable lines on the vertical surface. When spraying commenced, the pooling affect was observed making line resolution impossible, as can be seen in figure 4.48. The ECO spray and the air gun were very similar based on their performance in this experiment. However, the air gun, based on the results of the t-test, was better at resolving blood lines, though not significantly. Both the air gun and the ECO spray performed better than the ABA sprayer, though not significantly better than the ABA finer sprayer. The ABA spray performed better than the ABA finger sprayer. Below are images from one of the 3mm samples for each of the different spray types.
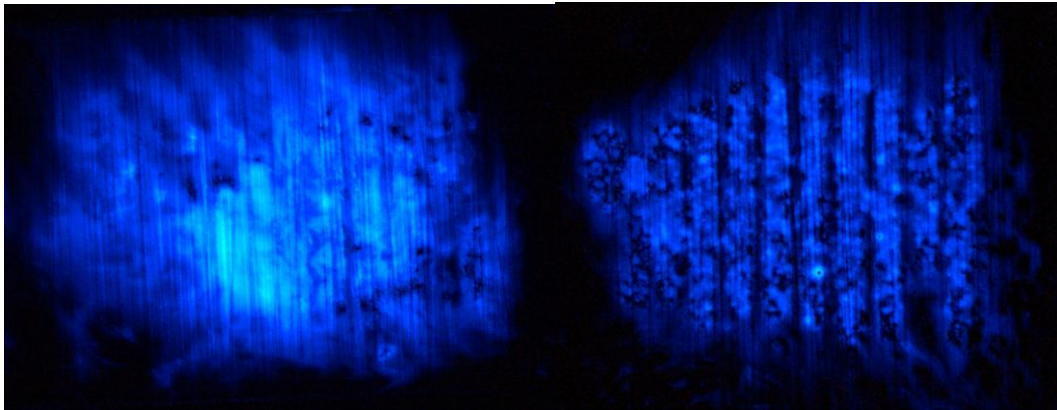
3mm Spray Pump

3mm ABA Finger
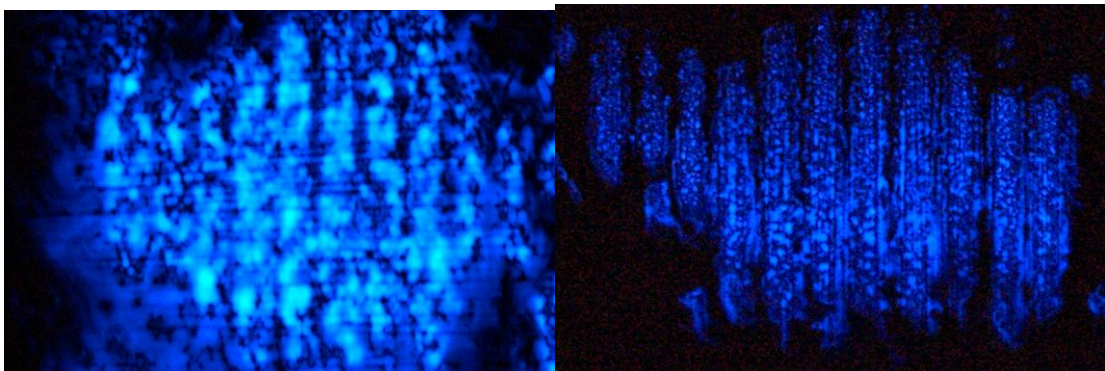
3mm ECO Spray
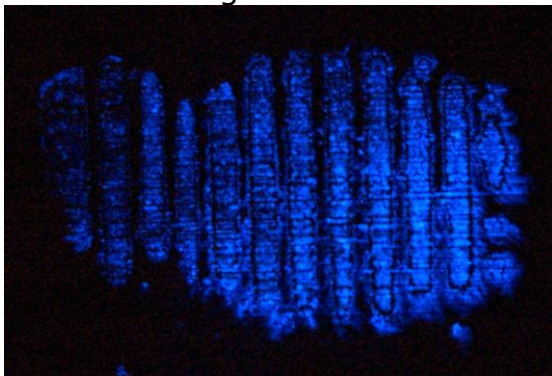
3mm Spray Gun

Figure 4.47: 3mm- Images showing the ability of each of the different spray types to reduce blood pattern distortion on horizontal surfaces

2mm

Evaluation of the 2mm samples was undertaken similarly to the 3mm samples. Multiple regression was then undertaken to statistically show whether one spray type out performed the other based on the difference values. Below is a table giving the results of the 2mm line samples.

	Eco Spray		Aba Spray		Aba finger		Sprayer	
	Lines	Spaces	Lines	Spaces	Lines	Spaces	Lines	Spaces
Air Gun	0.0132	0.457	0.0566	0.000208	-	-	-	-
Eco Spray			0.511	0.000214	-	-	-	-
Aba Spray					-	-	-	-
Aba Finger	-	-	-	-	-	-	-	-

Table 4.15: The *p*-values for the multiple regression testing each spray type against the others.

Only three out of the five spray types managed to resolve the 2mm lines. These were the ABA spray, ECO spray and the spray gun. The pump sprayer and the ABA finger caused pooling of the reagent on the sample, preventing the measurement of the lines and spaces. The air gun performed significantly better than both the ECO spray and the ABA spray in the resolution of the lines. The airgun was only significantly better than the ABA spray at resolving spaces. The ECO spray was not significantly better at resolving lines than the ABA spray but was significantly better at resolving spaces. Based on the p-values and the t-statistics for each spray type which managed to resolve the 2mm blood lines, the air gun performed the best, the ECO spray was next best at resolving lines after luminol application and the ABA spray was least best. Below are images from one of the 2mm samples for each of the different spray types that managed to resolve lines and spaces from at least one sample.
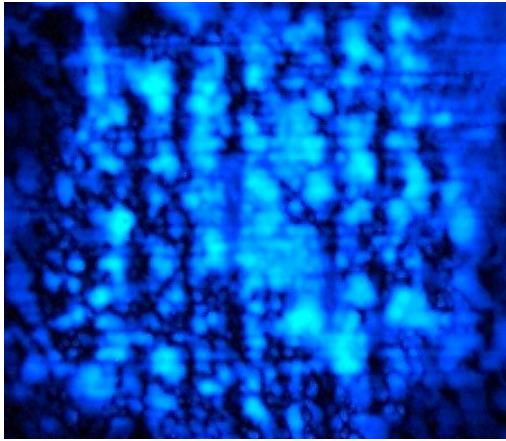
2mm ABA Spray
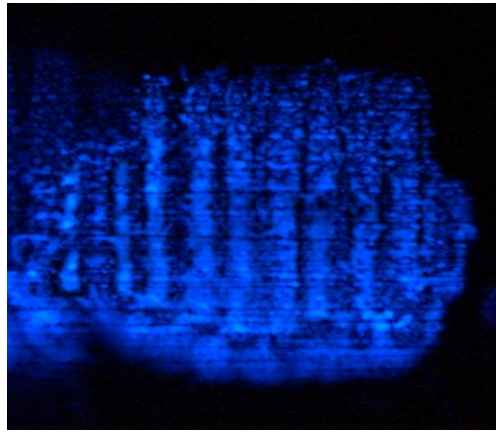
2mm ECO spray
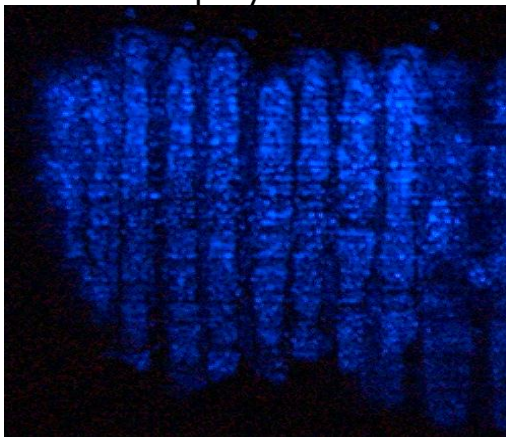
2mm Spray Gun

Figure 4.48: 2mm- Images showing the ability of each of the different spray types to reduce blood pattern distortion on horizontal surfaces

1mm

Evaluation of the 1mm samples was undertaken similarly to the 2mm and 3mm samples. Multiple regression was then undertaken to statistically show whether one spray type out performed the other based on the difference values. Below is a table giving the results of the 1mm line samples.

	Eco Spray		Aba Spray		Aba finger		Sprayer	
	Lines	Spaces	Lines	Spaces	Lines	Spaces	Lines	Spaces
Air Gun	0.0193	0.0880	-	-	-	-	-	-
Eco Spray			-	-	-	-	-	-
Aba Spray	-	-	-	-	-	-	-	-

| Aba Finger | - | - | - | - | - | - | - | - |

Table 4.16: The p-values for the multiple regression testing each spray type against the others.

Only two spray types out of the five participating in this experiment managed to resolve the 1mm lines. The air gun performed better that the ECO spray and was significantly better than the ECO spray at resolving the 1mm lines but not at resolving the 1mm spaces.

1mm Air Gun   1mm ECO spray
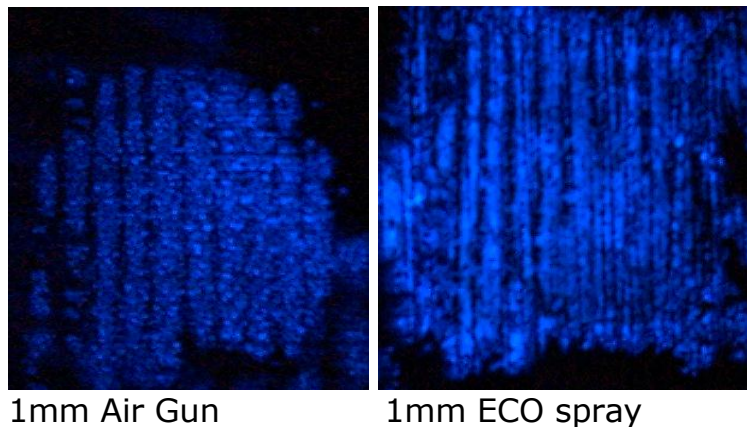
Figure 4.49:   1mm-Images showing the ability of each of the different spray types to reduce blood pattern distortion on horizontal surfaces

4.7   Morphoanalytical   Study-   Fixative/Shear Thinning Agent

This next section of experimental results sought to evaluate how each fixative/shear thinning agent, when utilised with Grodsky luminol, was able to retain a blood pattern. That is, to investigate which fixative/shear thinning agent is best for preventing luminol destruction to blood patterns on vertical and horizontal non-porous surfaces.

4.7.1 Vertical Non-Porous Surface

The results below show the ability of the different spray types to prevent blood pattern destruction on vertical, non-porous surfaces. The different fixatives were scored by the following criteria: The letter N was assigned to samples in which lines of the particular sample could not be resolved. The letter Y was assigned to

samples in which lines of the particular sample could be resolved. The letter r was assigned if the image showed signs of luminol running down away from the lines. The letters Ys were assigned if the lines in the image could be resolved but the lines were smeared or otherwise not well defined.

	3mm			2mm			1mm		
	Tile 1	Tile 2	Vinyl	Tile 1	Tile 2	Vinyl	Tile 1	Tile 2	Vinyl
No fixative	Nsr	Nsr	Nsr	Nsr	Nsr	Nsr	Nsr	Ns r	Nsr
ABA fix	Y	Y	Y	Y	Y	Y	Y	Y	Y
Xanthan gum	Y	Y	Y	Y	Y	Y	N	N	N
Alcohol	Y rs	Y rs	Yrs	N r	Nr	Nr	Nr	Nr	Nr
Zinc 1	Ysr	Ysr	Ysr	Ysr	Ys	Nsr	Nsr	Nsr	Nsr
Zinc 2	Ysr	Ysr	Ysr	Ysr	Ysr	Ysr	Ysr	Ysr	Ysr
ABA fix + Z2 + Eco	Y	Y	Y	Y	Y	Y	Y	Y	Y
ABA fix + Z2 + Eco	Y	Y	Y	Y	Y	Y	Y	Y	Y

Key:
N= cannot distinguish lines
Y= can distinguish lines
r = "running"
s= "smearing"

Table 4.17: Results of the effect of the different spray types on blood patterns on the two different substrates

From the above table it can be seen that luminol sprayed with the spray gun for 3 seconds without a fixative or shear thinning agent caused running when sprayed on bloody lines and the lines could not be resolved. An example of the illegibility of 3mm blood lines when no fixative is used is shown in figure 4.50. The ABA fix shear thinning agent and the two combination treatments (xanthan gum + Z2+ ECO and xanthan gum + Z2 + ABA spray) proved very effective at resolving all the samples with no running or smearing observed. The xanthan gum resolved lines of 3mm and 2mm but failed to resolve samples with 1mm lines. Although no running was observed in the samples sprayed with xanthan gum containing luminol, there was smearing. An example of this can be seen in figure 4.50 where the lines can be

resolved though they are not well defined. The alcohol fixative managed only to resolve lines of 3mm width and also failed to prevent running and smearing occurring, as seen in figure 4.50. Resolution in this picture is only possible near the top of the image. The zinc 1 fixative resolved the 3mm line samples, two out of the three 2mm line samples but none of the 1mm line samples. In all samples a characteristic running and smearing could be seen, which is demonstrated in figure 4.50. The zinc 2 fixative resolved the lines of all the samples, though running was still observed as seen in figure 4.50. Also observable in figures 4.50 and 4.50 is the apparent decrease in chemiluminescence compared to the other images.

3mm No Fixative   3mm Alcohol

3mm ABA fix   3mm Xanthan Gum

3mm Z1   3mm Z2

1mm ABA Fix   1mm Xanthan Gum

1mm Z1   1mm Z2
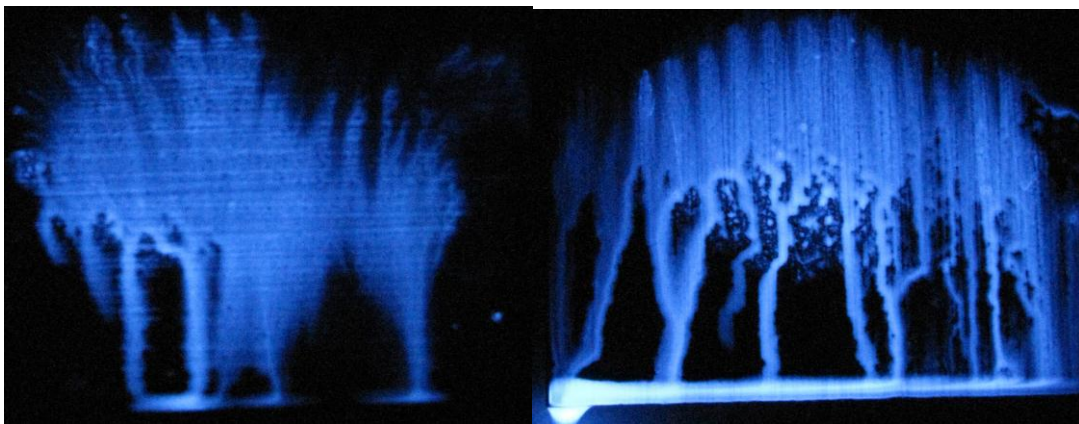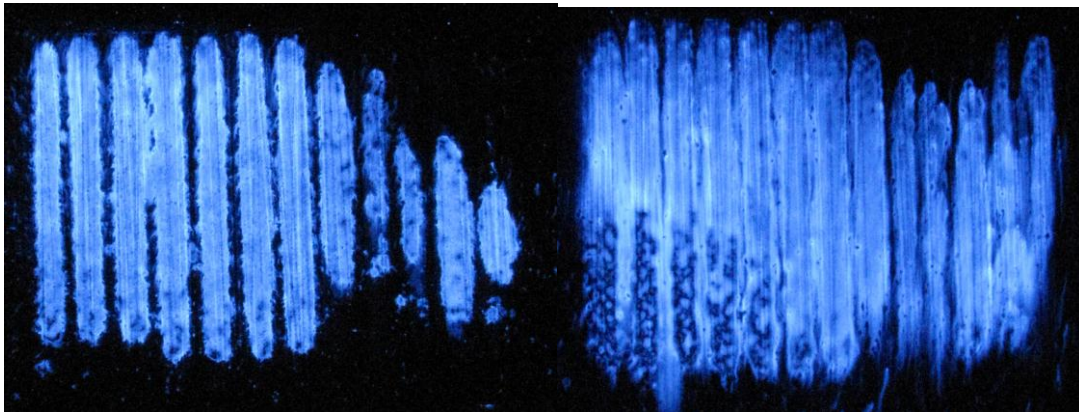
Figure 4.50: Images showing the ability of each of the different fixatives/shear thinning agents to reduce blood pattern distortion on vertical surfaces

4.7.2 Horizontal Non-Porous Surface

The table below displays the abilities of the different fixatives/shear thinning agents to prevent blood pattern destruction on horizontal non-porous surfaces. The values obtained from measuring each blood line and space before and after treatment were recorded onto an EXCEL spreadsheet. The difference between these two measurements was then obtained to evaluate the extent of distortion occurring to the lines after luminol treatment. Multiple regression was then undertaken to statistically show whether one fixative/shear thinning agent out performed the other based on the difference values. Below is a table giving the results of the 3mm line samples.

| 3mm	X+Z2+Aba	Z1	Z2	Abafix	Xanthan	Alcohol	No fixative
X+Z2+ Eco	0.0247	4.95E-7	0.00660	2.68E-14	9.61E-13	0.104E-3	-
X+Z2+ Aba		0.00854	0.391	3.81E-11	2.3E-9	0.0229	-
Z1			0.234	7.86E-9	3.27E-7	0.592	-
Z2				4.471E-5	1.02E-3	0.592	-
Abafix					0.235	1.75E-6	-
Xanthan gum						2.94E-3	-
Alcohol							-

Table 4.18: The *p*-values for the multiple regression testing each fixative/shear thinning agent against the others.

From the p-values listed in the above table, it can be seen that Xanthan gum with the zinc 2 fixative sprayed via the ECO spray performed significantly better in this experiment by having the least difference between the thickness of the 3mm bloody lines before treatment and after treatment with luminol. The next best performance was the xanthan gum combined with the zinc 2 fixative sprayed via the ABA spray. This combination performed significantly better than ABA fix, xanthan gum, the

alcohol fixative and the zinc 1 fixative. However, this combination did not prove to be significantly better than the Z2 fixative. The Z2 fixative performed the next best and was significantly better than ABA fix and the xanthan gum shear thinning agents but not significantly better than the alcohol or Z1 fixative. The next best performing fixative was the alcohol fixative which was significantly better than the ABA fix and xanthan gum shear thinning agents at resolving the 3mm lines but not significantly better than the Z1 fixative. The Z1 fixative performed significantly better than the ABA fix and xanthan gum shear thinning agents. The ABA fix performed better than the xanthan gum based on the results of the t-test, however not significantly.

Below are some of the images pertaining to this experiment.

3mm No Fixative   3mm Alcohol

3mm ABA Fix   3mm Xanthan Gum

3mm Z1   3mmZ2

3mm Xanthan + Z2 + ABA Spray   3mm Xanthan + Z2+ ECO spray
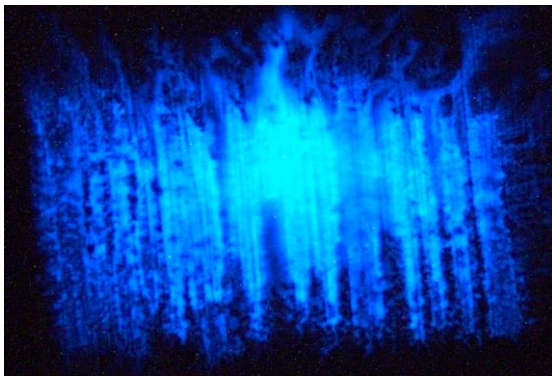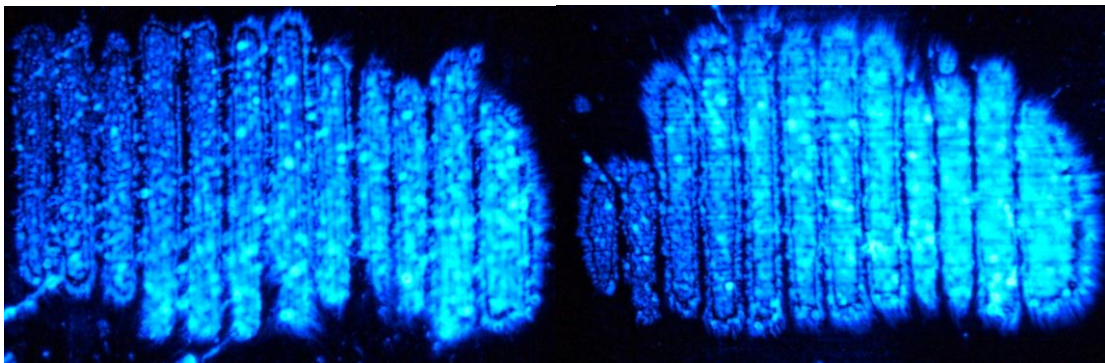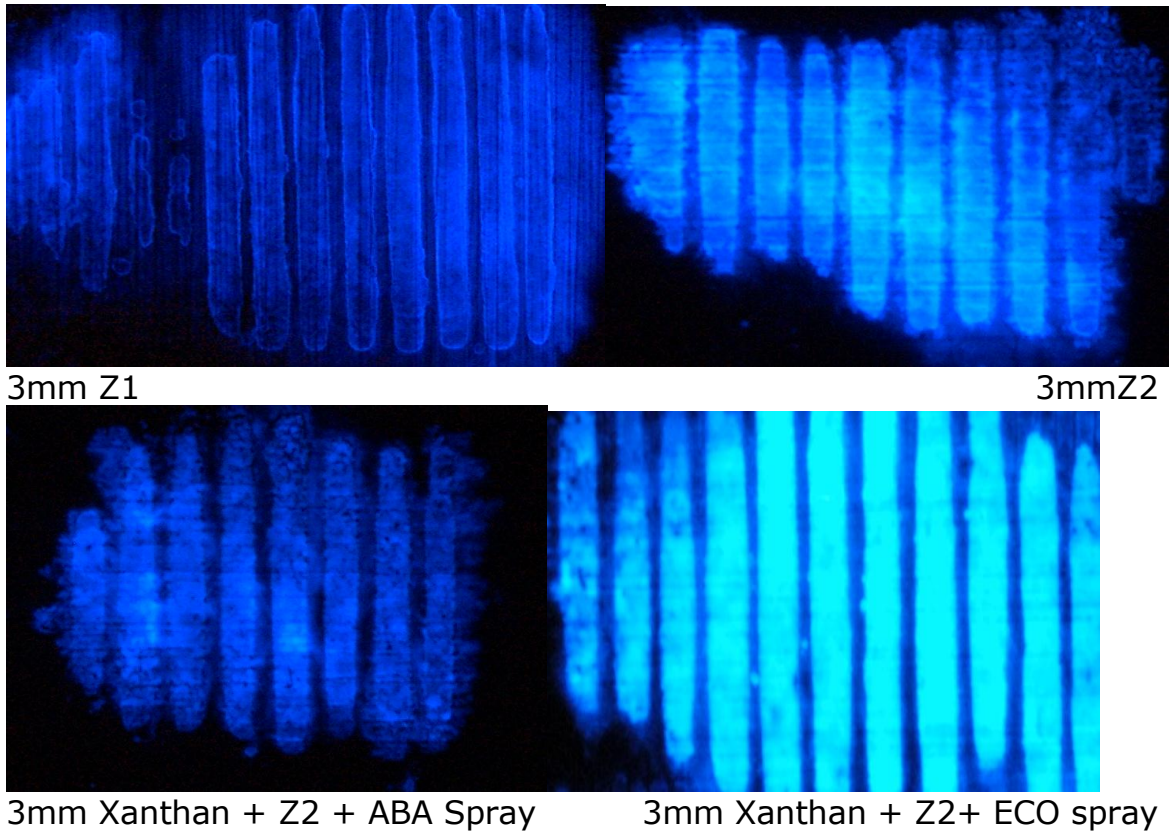
Figure 4.51: 3mm- Images showing the ability of each of the different fixatives/shear thinning agents to reduce blood pattern distortion on horizontal surfaces

2mm

Evaluation of the 2mm samples was undertaken similarly to the 3mm samples. Multiple regression was then undertaken to statistically show whether one fixative/shear thinning additive out performed the other based on the difference values. Below is a table giving the results of the 2mm line samples.

	X+Z2+Aba	Z1	Z2	Abafix	Xanthan	Alcohol	No fixative
X+Z2+Eco	0.961	0.108	0.985	1.66E-5	-	0.387	-
X+Z2+Aba		0.0987	0.972	3.08E-6	-	0.364	-
Z1			0.0764	2.63E-5	-	0.489	-
Z2				5E-8	-	0.338	-
Abafix					-	4.83E-6	-

Xantha n gum	-	-	-	-	-		-
Alcohol							-

Table 4.19: The *p*-values for the multiple regression testing each fixative/shear thinning agent against the others.

The xanthan gum failed to resolve the 2mm line samples. From the p-values listed in the above table, all the fixatives/shear thinning agents performed significantly better than ABA fix and Z2 performed significantly better than Z1. The rest could not be significantly separated. Based on the t-statistics from the multiple regression, the xanthan gum, Z2, ECO spray combination performed the best followed by the xanthan gum, Z2, ABA spray, Z2, the alcohol fixative, Z1 and ABA fix, respectively. Below are some of the images pertaining to this experiment.

2mm No Fixative   2mm Alcohol

2mm ABA fix   2mm Xanthan Gum

2mm Z1   2mm Z2

2mm Xanthan + Z2+ ABA spray   2mm Xanthan + Z2 + ECO spray
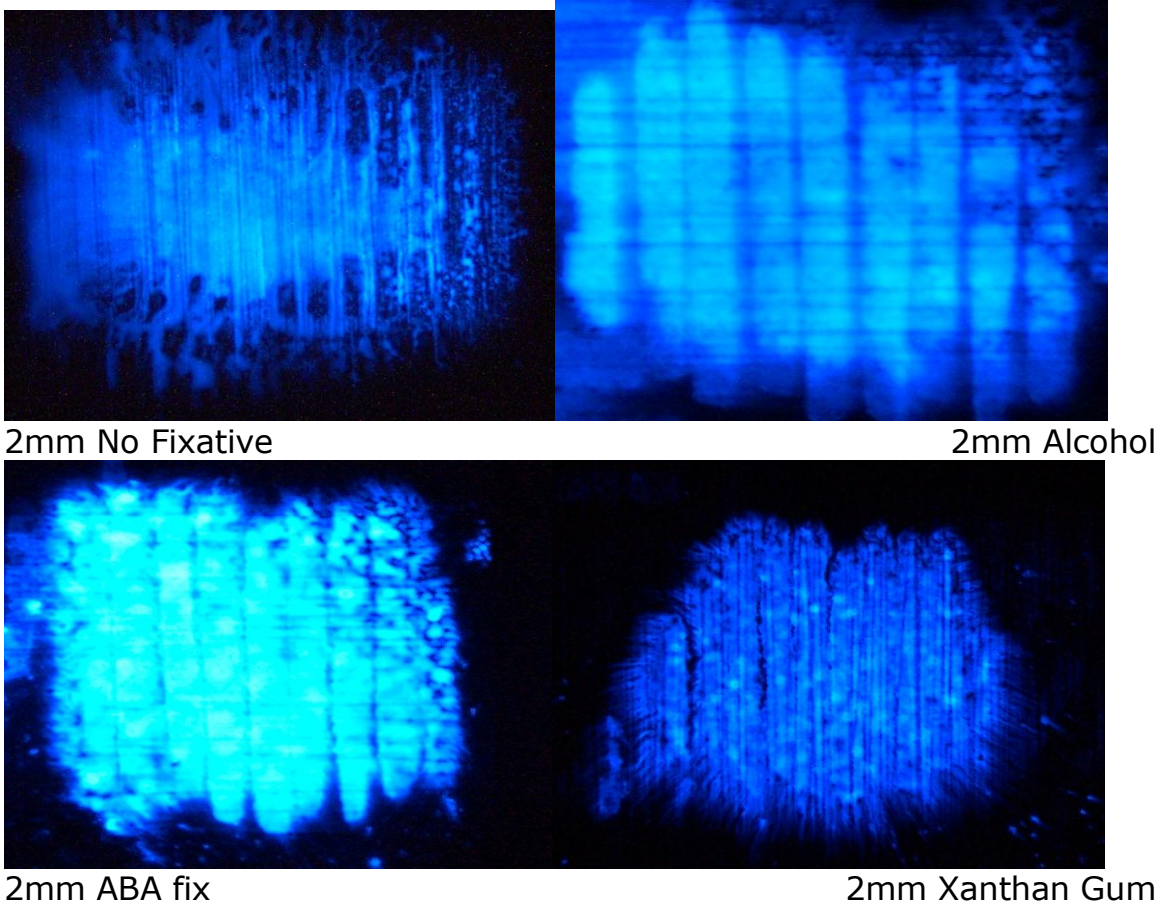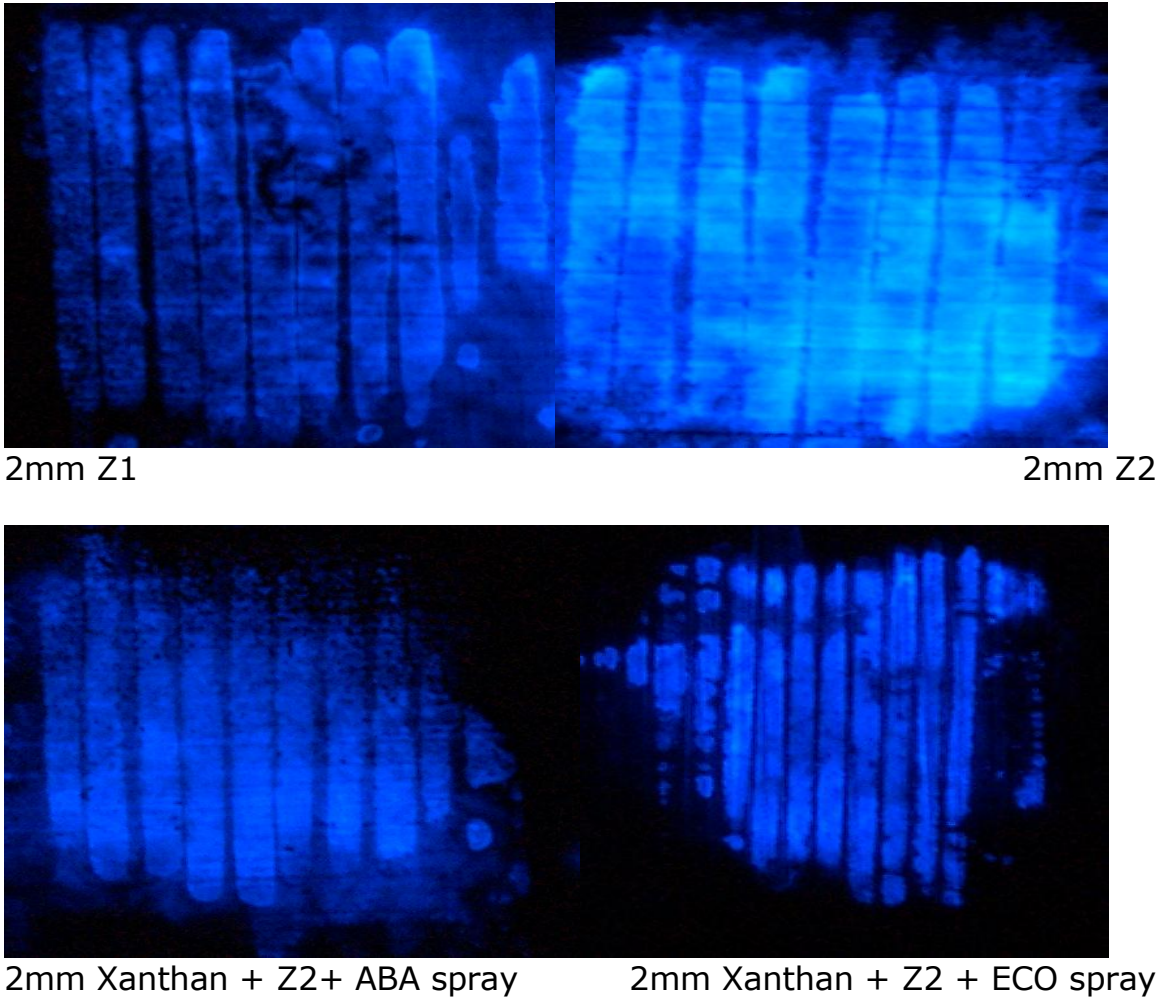
Figure 4.52: 2mm- Images showing the ability of each of the different fixatives/shear thinning agents to reduce blood pattern distortion on horizontal surfaces

1mm

Evaluation of the 1mm samples was undertaken similarly to the 2mm and 3mm samples. Multiple regression was then undertaken to statistically show whether one fixative/shear thinning additive out performed the other based on the difference values. Below is a table giving the results of the 1mm line samples.

	X+Z2+Aba	Z1	Z2	Abafix	Xanthan	Alcohol	No fixative
X+Z2+Eco	0.0344	0.305	0.327	0.00658	-	0.0108	-
X+Z2+Aba		0.231	0.133	0.0392	-	0.0192	-

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Z1	0.753	0.0229	-	0.0229	-	
Z2		0.00810	-	0.0210	-	
Abafix			-	0.974	-	
Xanthan gum	-	-	-	-	-	-	-
Alcohol					-	

Table 4.20: The *p*-values for the multiple regression testing each fixative/shear thinning agent against the others.

The xanthan gum failed to resolve the 1mm line samples. From the p-values listed in the above table, all the fixatives/shear thinning agents performed significantly better than ABA fix and the alcohol fixative. The alcohol fixative and ABA fix performed quite similarly with the alcohol fixative, having the slightly better performance based on the average values. Again the xanthan gum, Z2, ECO spray combination performed the best followed by the xanthan gum, Z2, ABA spray combination, Z2 and Z1 respectively.

Below are some of the images pertaining to this experiment.

1mm No Fixative   1mm Alcohol

1mm ABA fix   1mm Xanthan

1mm Z1   1mm Z2

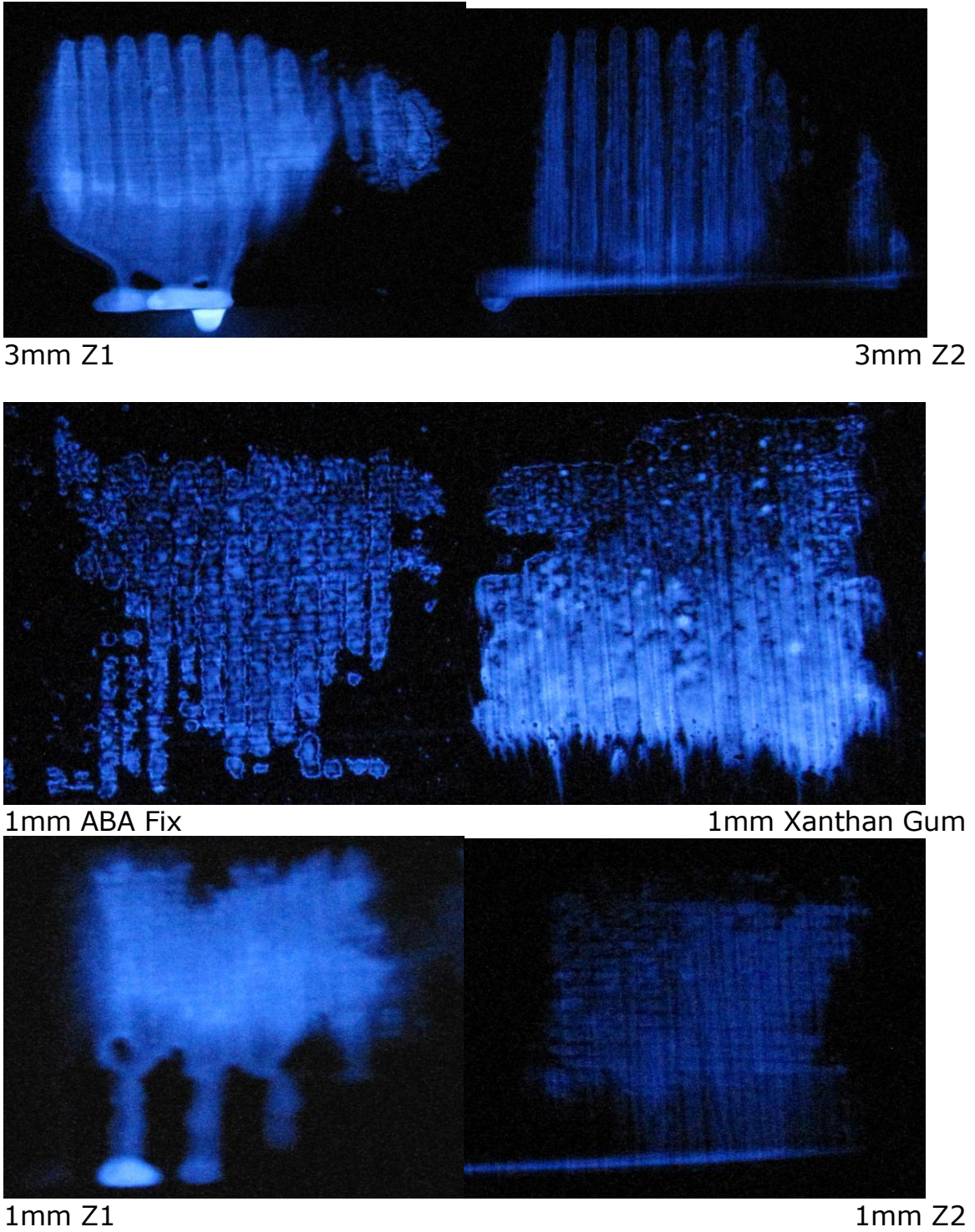1mm Xanthan + Z2+ ABA Spray   1mm Xanthan+ Z2+ ECO Spray
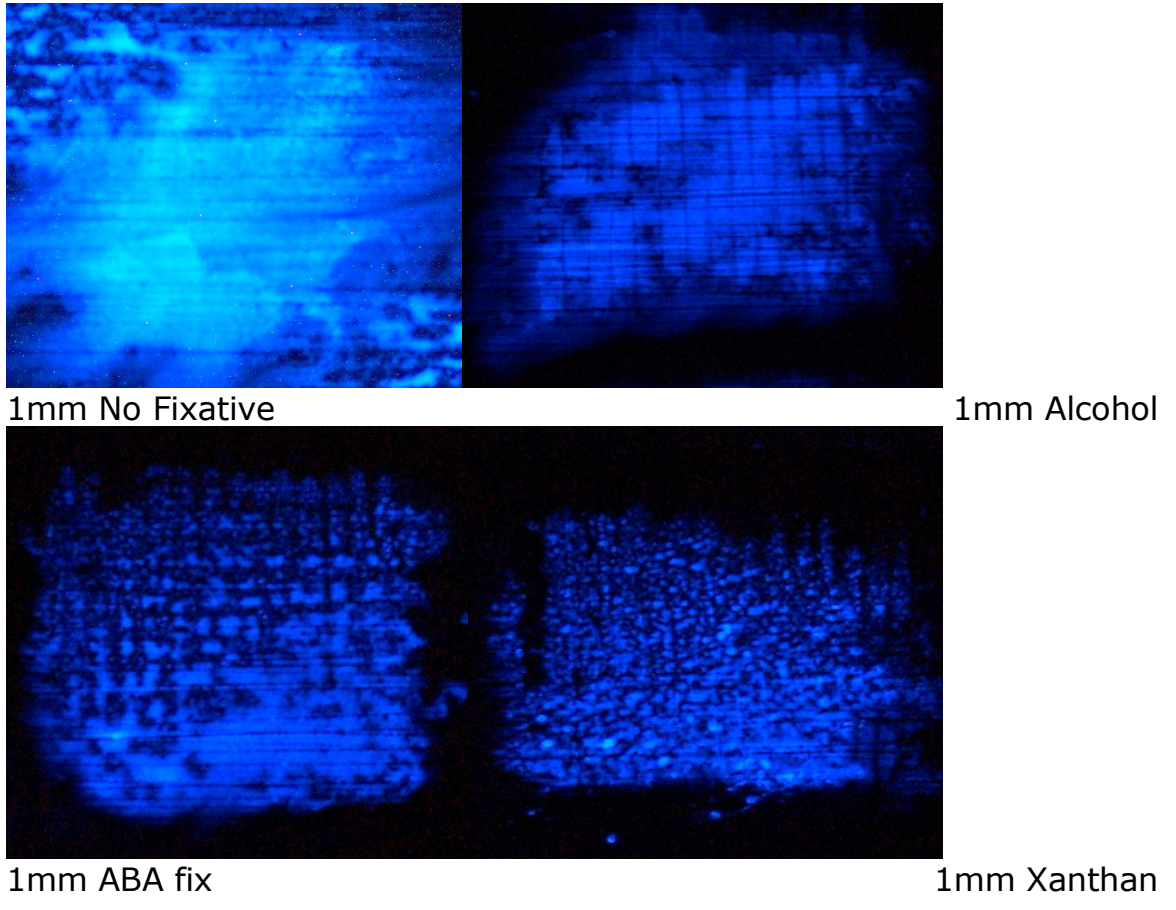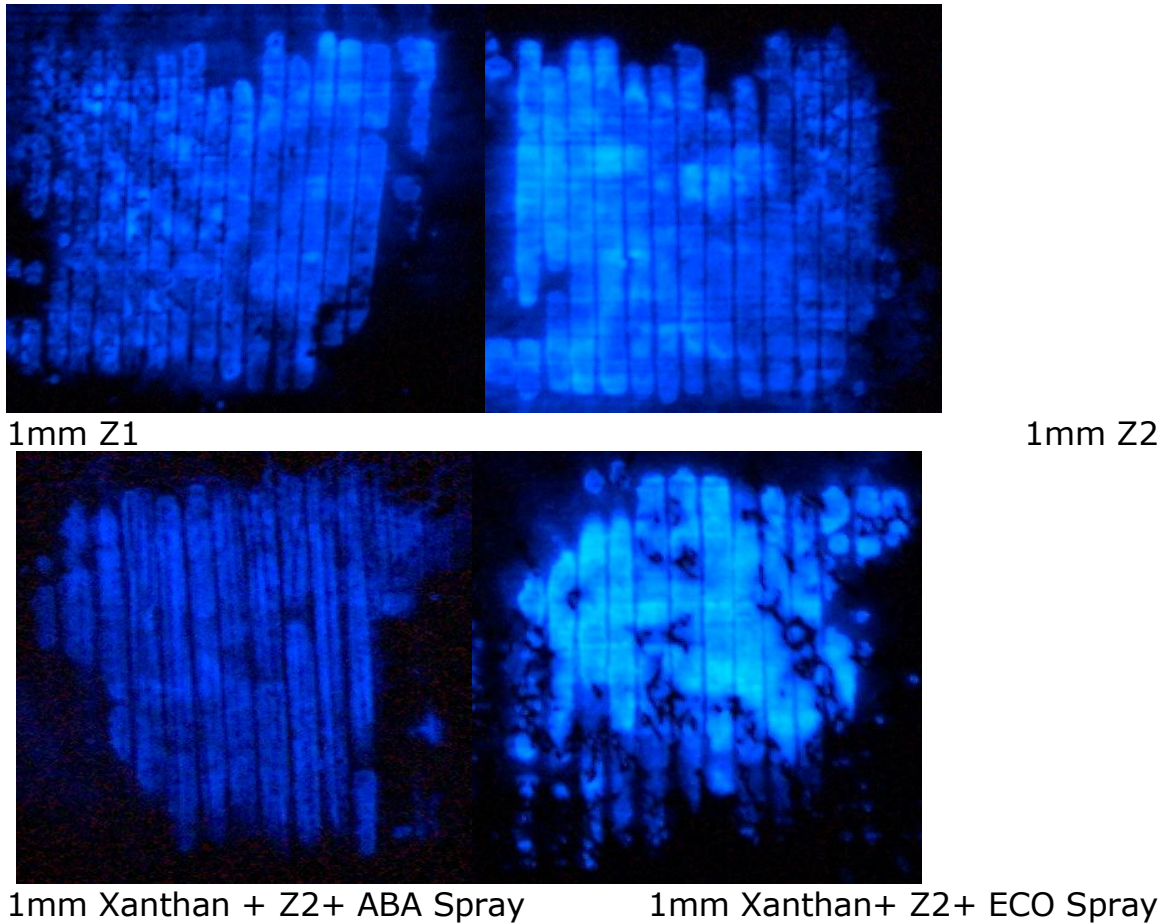
Figure 4.53: 1mn- Images showing the ability of each of the different fixatives/shear thinning agents to reduce blood pattern distortion on horizontal surfaces

3mm spaces

The 3mm spaces data was then analysed similarly to the 3mm line data. Below is a table giving the results of the multiple regression.

	X+Z2+Aba	Z1	Z2	Abafix	Xanthan	Alcohol	No fixative
X+Z2+Eco	0.0134	0.000491	0.328	3.11E-27	6.28E-16	0.00324	-
X+Z2+Aba		0.284	0.773	1.61E-21	2.15E-12	0.105	-
Z1			0.318	1.4E-19	2.24E-11	0.276	-
Z2				5.37E-12	1.55E-6	0.241	-
Abafix					0.724	1.15E-8	-

	X+Z2+Aba	Z1	Z2	Abafix	Xanthan	Alcohol	No fixative
Xanthan gum						3.95E-5	-
Alcohol							-

Table 4.21: The *p*-values for the multiple regression testing each fixative/shear thinning agent against the others.

The results in the above table of the 3mm spaces multiple regression are similar to the results obtained from the 3mm line multiple regression. The xanthan gum, Z2, ECO spray combination performed the best and was significantly better than all the other fixatives/shear thinning, excluding Z2. The rest of the fixatives/shear thinning agents were significantly better than the ABA fix and xanthan gum shear thinning agents. Based on the results of the t-test, the xanthan gum, Z2, ABA spray combination performed next best followed by Z2, Z1, alcohol fixative, ABA fix and xanthan gum, respectively.

2mm space

The 2mm spaces data was then analysed similarly to the 3mm line data. Below is a table giving the results of the multiple regression.

	X+Z2+Aba	Z1	Z2	Abafix	Xanthan	Alcohol	No fixative
X+Z2+Eco	0.994	0.278	0.903	4.4E-6	-	0.0219	-
X+Z2+Aba		0.278	0.968	8.92E-6	-	0.0253	-
Z1			0.201	6.04E-5	-	0.138	-
Z2				2.06E-6	-	0.0143	-
Abafix					-	0.00835	-
Xanthan gum	-	-	-	-	-	-	-
Alcohol					-		-

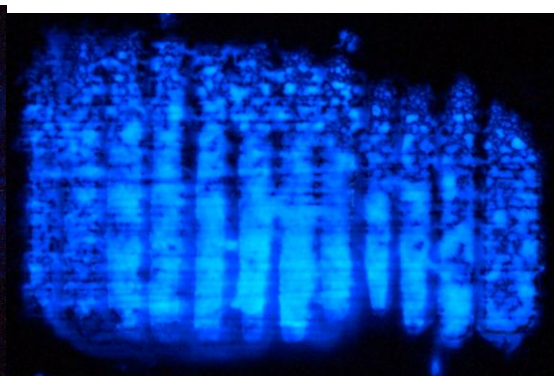**Table 4.22: The *p*-values for the multiple regression testing each fixative/shear thinning agent against the others.**

The results in the above table of the 2mm spaces multiple regression are similar to the results obtained from the 2mm line multiple regression. Xanthan gum failed to resolve the 2mm lines in all of the samples. The xanthan gum, Z2, ECO spray combination performed the best and was significantly better than the ABA fix and the alcohol fixative. The rest of the fixatives/shear thinning agents were significantly better than the ABA fix shear thinning agent and all the reagents except for Z1 performed better than the alcohol fixative. Based on the results of the t-test, the xanthan gum, Z2, ABA spray combination performed next best followed by Z2, Z1, alcohol fixative and ABA fix, respectively. The xanthan gum, Z2, ECO spray combination, the xanthan gum, Z2, ABA fix combination and the Z2 fixative performed very similarly.

1mm space

The 1mm spaces data was then analysed similarly to the 1mm line data. Below is a table giving the results of the multiple regression.

	X+Z2+Aba	Z1	Z2	Abafix	Xanthan	Alcohol	No fixative
X+Z2+ Eco	0.000971	0.0019	0.966	0.000876		0.000876	-
X+Z2+ Aba		0.0357	0.277	0.00437		0.277	-
Z1			0.137	0.0018		0.181	-
Z2				1.88E-6		1.88E-6	-
Abafix						0.00963	-
Xanthan gum	-	-	-	-	-	-	-
Alcohol					-		-

Table 4.23: The *p*-values for the multiple regression testing each fixative/shear thinning agent against the others.

The results in the above table of the 1mm spaces multiple regression are similar to the results obtained from the 1mm line multiple regression. Xanthan gum failed to resolve the 1mm lines in any of the samples. The xanthan gum, Z2, ECO spray combination performed the best and was significantly better than the ABA fix, Z1 and the alcohol fixative. Based on the results of the t-test, the xanthan gum, Z2,

ABA spray combination performed next best followed by Z2, Z1, alcohol fixative and ABA fix, respectively. The xanthan gum, Z2, ECO spray combination and the Z2 fixative performed very similarly.

5. Discussion

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5.1 Blood Detecting Reagents

In this thesis five blood detecting reagents were compared in terms of their sensitivity, longevity of their reaction with blood, ability to preserve DNA and their ability to retain the morphology of blood patterns. Additionally, some non-experimental parameters regarding the economical and practical worth of the reagents were investigated. Four of the reagents were luminol based formulas, namely Bluestar Magnum, Lumiscene, Lumiscene Ultra and Grodsky, and one was a fluorescien based reagent, Hemascein. Descriptions of these can be found in section 2.4.

5.1.1 Sensitivity

This experiment was set up to mimic crime scene conditions while controlling for variables including the amount blood used, the amount of reagent delivered, the ambient light level and substrate type. All of these mentioned variables can greatly affect the light intensity of a chemiluminescent and fluorescent reaction. The amount of blood used was controlled by pipetting a known constant amount of blood onto the substrate. The substrate used was always the cotton/polyester fabric. The amount of reagent delivered was controlled by using a calibrated spray gun. The spray gun is not the common method of applying luminol at a crime scene but was deemed necessary in this experiment to ensure an equal amount of the reagent was delivered to each sample. Ambient light levels were controlled for by treating all samples in a dark, windowless room.

Many studies have utilised a subjective approach to assess the sensitivity of luminol and fluorescein reactions (section 1.4.4.1). In a subjective evaluation the results rely solely on the discrimination ability of the examiner and as such are subject to variations in the examiners judgement. The quantitative approach taken in this

experiment was deemed a more accurate and repeatable way to assess the light intensity of the reactions because the figures are directly comparable between the different reagents and not subjectively obtained. This quantitative approach to obtaining a measure of light intensity is discussed in detail in section 3.2.1. Accurate measurement of intensity by this method depends on the accurate collection of the emitted light. The emitted light was captured by a Nikon D100 digital camera. The camera has a digital sensor which captures the light and it is expected that the amount of light recorded by the camera is directly proportional to the amount of emitted from the reaction. However, there are two exceptions to this. The camera sensor can become saturated if the sensor reaches a maximum of light which can be recorded. This occurs because light is converted to an electrical signal by each pixel sensor. The maximum signal that a sensor can process before any signal above this value is processed the same is the sensitivity of the sensor. In this case, the camera will not record intensities of light higher than this maximum threshold (3,106). To avoid this, the experiments were started at a blood concentration of 1:5000. From experimental observation, light from the reactions at this concentration did not saturate the camera sensor. The second exception is that the sensor creates noise which, at lower intensities, can have a significant effect on the reading and introduce bias. Noise is particularly prevalent with increased ISO values. This makes the camera sensor more sensitive to light so that ambient light, even in a dark room, produces a weak signal (139). To reduce this effect, a threshold value was obtained by assessing the background intensity and subtracting this from the averaged intensity value of the reaction.

In addition to the quantitative analysis of the different reagents sensitivity was assessed using Matlab to process the images obtained. Three types of images were displayed in the results section of this thesis representing unaltered images, images processed in Matlab whereby a 3x3 median filter has been applied over the image and images further processed in Matlab whereby geometric averaging in a 15x15 area was undertaken. In these pictures it is apparent that much lower blood dilutions can be detected when the images were processed in Matlab compared to the unaltered images. The Matlab processed images provide a means of viewing reactions which are very faint and or invisible in unprocessed images.

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However, two of the reagents in this study, Lumiscene Ultra and Hemascein, produced Matlab images which were not representative of the reaction. The Lumiscene Ultra images however show bright spots which interfered with processing the pictures in Matlab. The explanation for these bright spots is unknown but Lumiscene Ultra may have been reacting with the substrate to a certain extent. This produced images where that falsely represented the true position of the bloodstain and existence of the reaction. This effect may also account for the higher intensity readings at blood concentrations of 1:500,000 and lower. The Matlab images produced from the Hemascein samples inaccurately described the shape and position of the blood spots at lower dilutions. This was probably due to background fluorescence which produced false positives that interfered with filtering and averaging leading to images that were not representative of the reaction. Steps were taken to reduce the effect of background fluorescence by methods described in section 3.2, though useable images were still unattainable. More research would be needed to ensure this method of viewing bloodstains can be successfully applied to fluorescent reactions.

Contrast between the amount of light emitted by the different reagents in this thesis depended largely on the concentration of the blood. At higher concentrations there was a profound difference in emitted light intensity while at lower concentrations this difference was less noticeable. This effect may be in part explained by the previous paragraph whereby even if a formula was to react more efficiently with haem than another, the restricted amount of haem available for the reaction would prevent a large difference in light intensity emission between the different formulas. This effect is most noticeable when Grodsky is compared with Lumiscene Ultra. Lumiscene Ultra had the highest average light intensity for the first blood concentration of 1:5000 at 146 arbitrary units (AU) while the Grodsky reagent produced a light intensity value of 15AU at the same concentration. However, as the concentration of blood was reduced, the difference between the two reagents was also reduced. At a blood concentration of 1:100,000, the average light intensity values for Lumiscene Ultra and Grodsky were 5.88 au and 1.58 au, respectively.

The shapes of the graphs in figures 4.1-4.5 show a decrease in light intensity of the reaction with decreasing blood dilutions which is not unexpected. At lower dilutions, there is less haem available to catalyse the luminol and fluorescein reactions thus less light is produced. The graphs also show that the relationship between chemiluminescence and blood concentration is non-linear. The slope of the graph is much steeper at higher blood concentrations than lower blood concentrations. This could be explained by an excess amount of heam available to catalyse the reaction at higher concentrations compared to the restricted amount of haem available at lower dilutions. Not all haem molecules react with luminol and fluorescein due to radiationless processes described in section 1.3.2. Therefore, when heam is restricted these effects may be more profound.

However, from the *p*-values of the multiple regression which eliminated the effect of concentration, Lumiscene Ultra, Lumiscene, Bluestar Magnum and Hemascien were significantly more sensitive than the Grodsky formula. The other reagents could not be significantly separated though the average intensity value for Lumiscene Ultra exceeded that of Bluestar Magnum which was higher than Lumiscene which in turn was higher than Hemascein, although not significantly.

From the intensity values and the images described above, a tentative limit of detection (LOD) was defined. Two LOD values were assigned by viewing the images for each reagent and deciding at which blood concentration a positive reaction is still definitely seen. The higher intensity LOD value related to the unprocessed images while the lower LOD value related to the Matlab images. These values were then plotted on a graph. The blood concentration before the reagent's intensity curve crossed the LOD line was considered that reagents LOD. These values were placed in a table. Both the graph and the table are in section 4.1.2. The LODs were tentatively assigned because there are many factors which may influence the sensitivity of these reagents including substrate structure, amount of blood or reagent and ambient light. However, in this situation the LODs provide a sensitivity comparison between each of the reagents. Unfortunately, the lower LOD value for Lumiscene Ultra and Hemascein may not be as accurate as the other

reagents due to the reasons stated above. Lumiscene Ultra, from table 4.3, shows a lower LOD value of 1:500,000 based on where the LOD line intersects the Lumiscene Ultra intensity curve in figure 4.12. From the Matlab produced images of the Lumiscene Ultra images, perhaps a lower LOD value of 1:100,000 and an upper LOD value of 2:100,000 should be assigned to the averaged LOD values from the Matlab processed images. This would mean that Bluestar Magnum, Lumiscene Ultra and Lumiscene are all inseparable in terms of sensitivity, which is in agreement with the p-values obtained in table 4.1. The LOD values for Hemascein Matlab processed images were unreliable for the reasons given above. However, the visual LOD for Hemascein was comparable to that of Bluestar Magnum, Lumiscene and Lumiscene Ultra with an upper LOD of 6:100,000 and a lower LOD of 4:100,000. The Grodsky formula was the least sensitive having a visual upper and lower LOD of 1:5000 and 1:10,000, respectively, and a Matlab upper and lower LOD of 6:100,000 and 4:100,000, respectively.

The method of utilising Matlab to visualise the exact location of luminol treated bloodstains described in section 3.2.1 could perhaps also be applied to crime scenes where luminol images are faint to see in unprocessed images. This could mean that blood can be detected at lower dilutions, essentially increasing the sensitivity of the reagent through the use of Matlab. An image of a luminol reaction can be captured and an area within the image defined as containing no blood can be assigned an intensity value to act as the threshold value. A 3x3 median filter can then be placed on the image to provide unaveraged images, defining the location of the reaction. When this is known, geometric averaging on the specific area where the reaction is taking place to further define the position and shape of the blood pattern. At a crime scene, images could be loaded onto a laptop and enhanced by the Matlab method outlined in section 3.2.1 to visualise bloodstains in case any additional analysis must be carried out on them. Also, images of a crime scene could be processed in the laboratory to produce enhanced images of the reaction for evidence,

5.1.2 Longevity of the Reaction

This experiment was set up as described for the sensitivity experiment and was designed to mimic a crime scene situation while also controlling for variables. These variables included the amount of blood in the sample, amount of reagent sprayed onto the sample, the ambient light level and the substrate type.

The results clearly showed that Hemascein had a significantly longer reaction than all of the reagents tested in this thesis. Hemascein was also the only fluorescein based reagent in this thesis, the other reagents were predominantly luminol based which explains the longer reaction time. Luminol light emission is chemiluminescent, as described in section 1.3.2 while light emission resulting from the reaction of Hemascein with blood is fluorescent. Chemiluminescent reactions are generally short lived (72) because when the excited triplet state species is formed, the excess energy is rapidly lost as this species returns to its ground state with the emission of light. Therefore, the light produced by a chemluminescent reaction is dependant on the number of molecules which reach this electronically excited triplet state. Emitted light ceases when all of the excited triplet state species, which in luminol is the 3-aminophthalate dianion (section 1.3.4), returns to the ground state. Once the aminophthalate dianion returns to the ground state it can not then emit any more light. However, the iron catalyst is not consumed in the reaction and if more luminol is added to the same bloodstain then chemiluminesence can again be observed. Therefore, the luminol reaction is limited by the ability of blood to catalyse hydrogen peroxide to provide the necessary oxidising agents for the reaction and the amount of luminol available to form the necessary excited triplet state intermediate. The length of the chemiluminescent reaction is dependant on the time it takes for all of the excited triplet state intermediates to return to their ground state.

The mechanism of light emission when a fluorescent reagent such as Hemascein reaction with blood is quite different. When fluoresin comes into contact with the oxidising agents produced by the reaction of hydrogen peroxide with iron in blood it is converted to fluorescein. Fluorescein is a fluorophore which means it can absorb light of a certain wavelength from an external source and then re-emit this light energy at a certain longer wavelength. Fluorescein will continue to fluoresce for as long as the external light source which excites the fluorescein is turned on or

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fluorescein is not consumed in the reaction. With the reaction of Hemascein with blood, fluorescein is not consumed in the reaction so therefore could theoretically fluoresce for as long as the external light source is on. However, there are certain other mechanisms whereby fluorescein can cease to fluoresce. Figure 4.16 shows the reaction profile for Hemascein. For the first 5 minutes of the reaction of Hemascein with blood the intensity of emitted light is relatively constant. There is a slight increase after 1 minute as fluorescence develops and more fluoresin is converted to fluorescein. Between this time and 15 minutes post inception the intensity of the reaction is greatly increased. This is perhaps due to a sudden increase in available hematin resulting from degradation of the erythrocytes in the sample converting more ferrous ions to Ferric ions. This could in turn catalyse more hydrogen peroxide molecules which could convert more fluroresin molecules to fluorescein. This increase in hematin could be a result of ultra violet damage to the erythocytes from the external light source used to excite the fluorescein molecules. This light source was left continuously on for the duration of the measured time interval for the Hemascein samples. UV light at the wavelength used in this experiment (415nm) is known to cause slight photohaemolysis to erythrocytes (136). However, long exposure of this UV light on a concentrated area of diluted blood could result in an increase in photohaemolysis. The next section of the graph in figure 4.16 shows a rapid decrease in the intensity of the reaction followed by a slower decrease. This could be due to photobleaching which is a phenomenon whereby fluorophores are lost though permanent photochemical destruction of the fluorescent molecule. Photobleaching of the fluorescein molecule can occur via two different mechanisms; the dye-to-dye (DD) and dye-to-oxygen (DO mechanisms. The DD mechanism involves the reaction between a fluorescein triplet and another fluorescein triplet or ground state dye molecule. This occurs when the concentration of fluorescin is much lower than the concentration of oxygen. If the concentration of fluorescein is high enough proximity induced reactions between the fluorescein dyes occurs which causes photobleaching. The DO mechanism occurs when the fluorescein concentration is less than the oxygen concentration and loss of fluorescence is caused by a reaction between an oxygen molecule and a fluorescein molecule. The rapid photobleaching may be caused by the DD mechanism because of the increased conversion of fluorescin to fluorescein. The

oxygen in the sample would be depleted and the fluoescein dye molecules would be in closer proximity to allow DD photobleaching. The slower decrease in intensity could be caused by the DO mechanism because by this time, the concentration of oxygen in the sample would probably be greater than the concentration of fluorescein.

For reasons probably due to the different constituents in the different luminol based formulas, Bluestar Magnum and Lumiscene had similar reaction lengths while Grodksy and Lumiscene Ultra had longer reaction lengths. The Grodsky and Lumiscene Ultra samples had a slower chemiluminescent decay than Bluestar Magnum and Lumiscene, as seen in figure 4.22. The intensity of chemiluminescent light at the start of the reaction for the Lumiscene Ultra samples was far greater than that of Grodsky, therefore the intensity values for the subsequent time intervals are greater up to 5 minutes after the reaction. The Grodksy formula has an intensity value much lower than the other reagents at the start of the reaction though after 1 minute the intensity values are comparable to those of Bluestar Magnum and Lumiscne. The different constituents of each of the luminol based formulas are not known, due to commercial restrictions, except for the Gordsky formula. Therefore a further examination of this potential an explanation of the results is not possible.

The Lumiscene Ultra reaction length seemed longer than the Grodsky reaction length but the same problems in the sensitivity experiment were encountered in this experiment. <mark>The Lumiscene Ultra images, however, show bright spots which interfered with processing the pictures in Matlab. The explanation for these bright spots is unknown but Lumiscene Ultra may have been reacting with the substrate to a certain extent. This produced averaged images that falsely represented the true position of the bloodstain and existence of the reaction.</mark> The unaveraged images, however, disclosed the true nature of the reaction better for comparison. This effect may also account for higher intensity values than would be expected from the images at times 15 minutes and longer. Because of the interference with the substrate for the Lumiscene Ultra samples, the LOD estimation in figure 4.23 should probably be adjusted to 4.5-5 minutes for the 10 au intensity value and 10 minutes

for the 5 au intensity value. This is the same LOD estimation numbers allotted to the Grodsky formula.

However, in all cases with the luminol based reagents, the intensity of the reaction was considerably more intense in the first 30 seconds of the reaction. Therefore, when photographing a crime scene, bloodstains treated with luminol based reagents should be photographed immediately to capture the highest intensity of the reaction. This is already recommended practice for photographing luminol reactions in forensic science (89)

For the Hemascein samples, background fluorescence seemed to develop extensively after 10 minutes of the reaction. Therefore, although the fluorescence was observed to increase after 10 minutes of the reaction so was the background fluorescence. Background fluorescence may lead to false positive identification of bloodstains. There was also some delay in the development of fluorescence as described above. One minute was generally enough time to allow for the increase in emitted light intensity. From the images and intensity values of the Hemascein reaction in this experiment, it is recommended that in casework situations, photographs of the Hemascein reaction are taken between one and ten minutes after the start of the reaction

5.1.3 DNA Analysis

The samples were set up as they were for the sensitivity and longevity experiment to mimic a crime scene while controlling which may affect DNA profiling such as the amount of blood used and the amount of reagent applied to the bloodstain. The samples were kept refrigerated for no longer than 30 days before quantification and STR profiling.

5.1.3.1 Quantification

The results showed that none of the reagents utilised in this study inhibited the PCR reaction based on the unimpaired profiling of the internal PCR control (IPC) sequence (134). The IPC values for all of the samples were within the accepted

level of 27.0+-0.5. German (138) found that Grodsky caused inhibition to PCR using the organic, forensicGEM™ and Maxwell™ extraction methods. However, he found that inhibitors could be removed from the sample using Microcon® 100 filters. The method utilised in this study to extract DNA was achieved through the DNA IQ™ System from Promega. This extraction method appears to also remove PCR inhibitors. Positive and negative controls were also included in the Identifiler kit to ensure the PCR reaction was working correctly. This meant that any impairment to profiling success of the reagents was due to degradation or insufficient amounts of template DNA.

The amount of template DNA extracted from each of the 1:10 bloodstain samples varied considerably from 0 ng/µl to 0.81 ng/µl (1.62ng). This may be due to uneven spreading of the white blood cells in the blood over the fabric as samples prepared for DNA analysis were hole punched from the main sample. Therefore, some punched out sample pieces may have contained more DNA than others. For the 1:10 bloodstain samples, one of the Bluestar Magnum and one of the Grodsky samples were undetected for an unknown reason. All of the Grodsky DNA quantification values showed the amount of DNA in the samples was very small compared to the other reagents with amounts of DNA detected in the range of 0.00218 ng/µl (0.0218ng) to 0.0102 ng/µl (0.136ng). This could be because the entire four Grodsky hole punched samples contained very small amounts of DNA or that the DNA was degraded by a chemical in the Grodsky formula. The study by German supports the latter theory whereby he found that the Grodsky formula degraded DNA while samples are in storage (138)

The 1:100 bloodstain samples showed typically low amounts of DNA ranging from 0 ng/µl to 0.008 ng/µl (0.8ng). Contrary to the 1:10 bloodstain samples as well, the Grodsky treated samples were not drastically smaller than the other reagents. One of the Bluestar Magnum samples contained undetected amounts of DNA for an unknown reason. Again, part of the bloodstained fabric was used in the quantification, therefore unequal amounts of DNA could be on each sample.

5.1.3.2 STR Profiling

The extent of degradation caused by each of the treatments was assessed in three different ways. Firstly, it is known that larger sized STRs are more susceptible to degradation than shorter STRs. Therefore, graphs were plotted displaying peak height from the STR profile vs STR size (figures 4.26-4.31). A linear line equation was then calculated in the form of y=mx +c. The slope of the line (mx) was then used as an indication of DNA degradation. Although the graphs show a wide spread of points, (figures 4.26-4.31) they were used as a comparison to the water treated control samples which showed a positive slope (figure 4.31). The line equation was only calculated for the 1:10 bloodstain samples though because the profiling success was reduced enough to see allele dropout (figure 4.39). The water samples had profiles similar to that of the positive control. The line equation was positively sloped and the longest STR fragments were 350bp meaning the larger STRs were not affected. The other treatments had slopes either slightly positive or slightly negative except for the Bluestar Magnum samples which were all negatively sloped. However, the longer STRs were still profiled. This reduction in longer STR peak height relative to the shorter STR peak heights could be an indication of DNA degradation. Examination of table 4.7 which displays a table of the p-values obtained from multiple regressions comparing the average slope value of the line equations for each of the reagents while eliminating the effects of DNA concentration shows that none of the reagents were could be significantly separated except for Hemascein outperforming Lumiscene Ultra. This result is surprising since Bluestar Magnum had the most negative line slope value. However, upon re-examination of figure 4.34, the data points are comparatively spread out which would reduce the difference between the Bluestar Magnum line slope value and the other reagents.

Examination of the 1:100 bloodstain scatter plots (figures 4.33-4.38) show that Lumiscene, Lumiscene Ultra and Grodsky all failed to successfully type the longer

STRs. This is especially apparent with the Lumiscene Ultra samples, which performed the worst. This may be indicative of DNA degradation due to higher concentration of the reagent. The water, Hemascein and Bluestar Magnum treated samples all managed to type the longer fragments. The overall peak height is reduced proportionally to the diminished amount of template DNA (figures 4.33-4.38)

The next measure of DNA degradation was overall peak height of the entire profile for each treatment. Table 4.48 displays the p-values from multiple regressions of peak height and eliminating the effect of DNA concentration. The peak heights of the STRs for the water treated samples were found to be significantly higher than the other treatments while Grodsky had the lowest peak heights. The lower peak heights obtained from the reagent treated samples, when compared to the water peak heights, might be indicative of DNA degradation since template DNA concentration was eliminated as a contributing factor through multiple regression.

However, when considering the 1:100 bloodstains (table 4.9), Bluestar Magnum had significantly higher peak heights than all the other treatments and the water peak heights were comparatively low. One explanation of this is DNA hydrolysis caused by water. The 1:100 bloodstain samples were more exposed to water than the 1:10 bloodstain samples. Therefore, the DNA in those samples would be more susceptible to hydrolysis. The reason why the Bluestar Magnum, Hemascein and Lumiscene samples experienced less reduction in peak height could be due to the different in pH of these reagents. The pH value of these reagents is higher than water so therefore could act as a buffer reducing DNA degradation through hydrolysis by water molecules.

The next measure of DNA degradation was to assess the profiling success for each of the treatments. This measure of degradation was the most relevant measure of what impact each reagent has on potential crime scene samples. Figure 4.40 shows the average profiling success for the 1:10 and 1:100 bloodstain samples. At the 1:10 blood dilution, 100% of the profile was obtained for most of the treatments. At 1:100 blood dilution, however, less of the profile was successfully typed. The average

percentage of alleles typed correctly was greatest in the water samples, which produced 80%. The reason for the profiling success being less than 100% for the water treated samples could be due to degradation of DNA over time in the presence of water through hydrolysis. Although, this could also be because of the low amounts of template DNA available for profiling (>0.8ng).

The profiling success for the rest of the reagents were lower than that of water, even though the average amount of template DNA in the water treated samples was less than that of the other reagents. This infers that there must be some mechanism which is responsible for the decrease in profiling success that interferes with the integrity of the DNA template. This observation could be described by any of the mechanisms described in section 1.4.4.3.1 including oxidative damage from oxidising agents or the alkaline conditions created by the luminol formula. Interestingly, the Grodsky formula did not compromise profiling success to the extent reported by Quinones or Almeida et al. For the total amount of DNA extracted from each of the 1:10 Grodksy samples, which was 0.136ng, 0.102ng and 0.0218ng, the percent of correctly typed STRs was 91.2%. For the 1:100 Grodsky the percentage was significantly reduced to around 30%, although the amount of DNA available for STR analysis was also reduced.

The reason for the decrease in profiling success for all of the reagents when comparing the 1:10 to 1:100 samples could be a combination of DNA degradation and insufficient template DNA. However, this does not explain how the Lumiscene Ultra samples, after achieving an average profiling success of 100% in the 1:10 samples, why the average profiling success was reduced to 17%. The amount of template DNA available for profiling was 0.02ng. Although this is a small amount of DNA, the amount of DNA available for the water samples was less and the profiling success of the water treated 1:100 samples was 80%.

==Also, results from previous studies (138) show that the Grodsky formula considerably hinders DNA profiling.== Although Grodsky hindered DNA profiling in this study, the effect was not as noticeable. This could be due to the DNA extraction

method in this thesis, Quantifiler® Real-time PCR, as it was not employed in the previous studies.

5.1.4 Morphoanalytical Study

The purpose of this experiment was to serve as a benchmark for the other morphoanalytical studies and to assess the ability of the different formulas to retain a blood pattern on vertical non-porous surfaces. Each reagent was sprayed for three seconds, using the spray gun, onto their corresponding samples. Three seconds was chosen because it was found experimentally that the spray gun is in itself reasonably efficient at preventing blood pattern distortion. This is probably due to the fine, nebulised mist produced by the sprayer. However, the spray gun is able to deliver a more precise and even spray than any other available method and it was important to keep the delivery volume consistent between the different reagents.

Consistent with other studies outlined in section 1.4.5.3, the luminol formulas poorly resolved the bloody lines and consistently caused a "running" effect. This running effect could be caused by the light emitting oxidised products formed when luminol is applied to a bloodstain diffusing away from the blood pattern or excess luminol reagent on the surface of the substrate self-chemiluminescing. These oxidised products do not absorb into the non-porous surface and instead, due to gravity, run down and away from the blood pattern. This either destroys or distorts the true location of the blood stains, depending on the extent of the running. Of the luminol based formulas, only Lumiscene Ultra appeared to maintain the integrity of the blood lines greater than a width of 2mm. The formula for Lumiscene Ultra is undisclosed therefore the reason for this observation is unexplained.

The Hemascein formula included the sheer thinning agent ABA fix as recommended by the manufacturer. Hemascein managed to resolve the bloody lines of all of the samples except one of the 1mm samples. However, if the photographs of the hemascein reaction were taken within 30 seconds of the application of the reagent then the lines were well defined. Otherwise, the lines became blurred together though did not "run" as was observed with luminol. This blurring of the lines could perhaps be due to a change in shear forces in the Hemascin/ABA fix once the

solution is applied to the bloodstain and the pseudoplastic nature of xanthan gum. Initially when the solution is sprayed onto a bloodstain the viscosity of the solution would be high because there would be no, or very little, shear stresses as the solution is deposited on the surface of the bloodstain. Because this is a vertical surface, gravitational forces work to pull the solution down. As the Hemascein/ABA fix solution is not entirely a solid, the liquid parts of solution begin to move. Molecules nearer the substrate would be moving slower than molecules nearer to the surface of the solution. This movement creates shear stress as parts of the solution would be moving parallel to other parts of the solution. An increase in shear stress decreases the viscosity of the solution. This makes the solution less viscous, increasing the ease of fluid movement and thus the lines start to blur together.

5.1.5 Practical and Economical Considerations

In determining which reagent should be employed for forensic science case work, other factors besides performance may influence the decision. These factors may be economical in nature such as the cost of a particular reagent or practical factors such as the ease of preparing and using the reagent in casework. The non-experimental parameters of economical or practical significance that were investigated were cost, toxicity, ease of preparation, practicality, shelf life (both activated and non-activated forms) and H_2O_2 content. Table 5.1 outlines the results of this investigation.

5.1.5.1 Cost

Because luminol is sometimes sprayed extensively in the search of latent blood then it may be desirable to employ a less expensive type of luminol. In table 5.2, the cost of each reagent kit and per millilitre of the working solution is presented. It should be noted that the prices below are not absolute prices and that prices may change at the companies' discretion.

In contrast to the other reagents, Grodsky luminol was not purchased as a kit but by bulk dry powders as prepared in this thesis. The cost listed below for Grodsky luminol was assessed by the cost to make 500ml of the luminol solution based on the total price of each powder and the amount used.

The Hemascein kit also comes with two ABA sprays valued separately at 14.95 USD each and xanthan gum which is included in the total price outlined in table 5.2. The price of the ABA sprays and xanthan gum cannot simply be subtracted from the total price to give the price of the reagent however because the reagents cannot be purchased separately to the whole kit.

The least expensive reagent tested in this thesis was Grodsky. Although the Lumiscene and Lumiscene Ultra kits are of a similar price, Lumiscein works out less expensive in terms the amount of working solution which can be prepared. Bluestar Magnum is the most expensive reagent in term the amount of working solution which can be prepared. The price of the Hemascien kit is within the price range of the Lumiscene Ultra kit, when comparing the amount of working solution which can be prepared. However, as mentioned above, the Hemascein kit comes with two ABA sprays and xanthan gum.

5.1.5.2 Toxicology

Knowing the toxicology of a reagent is important for the health and safely of the persons preparing and using that reagent. Also, it is important to know if any specific post-cleanup after an investigation needs to be performed before the scene or object is released back to the public. All of the reagents, to a certain extent, contain some constituents which are toxic. The health and safety data on luminol and its counterparts is described in detail in section 1.4.6.

The Bluestar Magnum, Lumiscene and Lumiscene Ultra working solutions contain sodium hydroxide and hydrogen peroxide which are corrosive, irritating to eyes and skin and may cause buns. The Hemascein method uses hydrogen peroxide which is classed as an irritant and is irritating to skin and eyes. Fluorescein itself is non-toxic and is used in many other fields in science including opthamology.

The risk of compromising health by the use of these reagents can be reduced when certain protocols are followed. One such protocol is the wearing of personal safety equipment such as gloves, face mask and safety glasses.

5.1.5.3 Ease of Preparation

Preparation time and difficulty of preparing the working solutions for each of the reagents was assessed.

The Grodsky luminol method was the most time consuming to prepare out of the reagents used in this study yet was still easy to prepare. Grodsky luminol is made up initially as two separate solutions which are mixed together immediately prior to spraying onto suspected blood stains. Solution A contains 0.5g of 3-aminopthalhydrazide and 25g anhydrous sodium carbonate diluted in 250 ml of distilled water. Solution B contains 3.5 g of sodium perborate diluted in 250 ml of distilled water. Both solutions are agitated separately until the respective powders have dissolved and then are mixed together prior to use. Solutions A and B are can be prepared in a lab and are mixed together at a crime scene or prepared at a crime scene.

The Hemascein reagent was the next hardest to prepare. 5ml of distilled water is added to the hemascein powder vial and is mixed. This is called the hemacein stock solution. 1ml of this solution per 100 ml of distilled water is mixed in one sprayer. This is the hemacein working solution. Hydrogen peroxide is then placed in a separate sprayer and diluted to 1-3%. The two solutions are sprayed separately onto bloodstains. First the working solution, then the hydrogen peroxide.

Lumiscene and lumiscene ultra were very easy to prepare. The activation tablets are added to the water based lumiscene stock solution and is shaken gently for 1 minute after 5, 10 and 15 minutes to activate the lumiscene. The lumiscene is then poured in a spray bottle.

Bluestar magnum was the easiest to prepare. The Bluestar forensic magnum chemiluminescent solution is poured into a spray bottle. Three activation tablets (oxidizing tablets) are added and dissolved by gentle stirring until the activation tablet is dissolved.

However, the ease of tablet based methods may also be a disadvantage. With tablet based methods, modification of the solution to suit different situations is not possible. This is an advantage of the Grodsky formula. One example of this is if faced with a very high background chemiluminescence, it may be desired to reduce the concentration of luminol to reduce this effect (113).

5.1.5.4 Shelf Life

The lengths of time that each of the reagent remains viable in both the unactivated and activated forms are displayed in table 5.1. As seen in this chart Hemascein and Grodsky have the longest unactivated shelf life of all the reagents. This is probably due to the dry powders preserving better than the liquid solutions of the other reagents. The Hemascein formulation vial is stable for 7 years at room temperature. The stock solution can be used for 30 days when stored at room temp under ordinary lighting conditions, 45 days when covered with aluminium foil and 15 months in a refrigerator. The working solution is stable for 28 days at room temp under normal lighting conditions, 64 days at room temp when covered with aluminium foil and 7 months if stored in a refrigerator. Bluestar Magnum, Lumiscene and Lumiscne Ultra all have a shelf live of three years in the unactivated form.

In the activated form, the shelf life of the luminol based reagents is significantly shorter than the shelf life of the Hemascein working solution. Grodsky, Lumiscene and Lumiscene Ultra had the lowest shelf life at four hours after the working solution was made. Blue star Magnum had a longer shelf life of 24 hours. However, the working solution of hemascein provided was by far the longest stability, being stable for up to seven months.

5.1.5.4 Hydrogen Peroxide Content

The H_2O_2 content for each of the reagents was calculated and added to the table 5.1 along with the source of the hydrogen peroxide. The source of hydrogen peroxide for Lumiscene, Lumiscene Ultra and Bluestar Magnum were activation tablets. The amount of H_2O_2 in the Bluestar Magnum tablets was unspecified by the manufacturer. The Lumiscene and Lumiscene Ultra working solutions claimed to have a final H_2O_2 concentration >12%. The source of hydrogen peroxide for the Hemascien samples is an individually sprayed solution of 1-3% hydrogen peroxide.

The total amount of hydrogen peroxide applied to a bloodstain depends on the amount of reagent sprayed because this would dilute the amount of hydrogen peroxide. The source of H_2O_2 for Grodsky luminol is sodium perborate. In aqueous solutions, sodium perborate breaks down into borate and H_2O_2. The amount of H_2O_2 produced is approximately half the original amount of sodium perborate.

5.1.5.5 Practicality

The practicality of utilising each reagent in casework was then assessed.

Firstly, while Bluestar Magnum, Lumiscene and Lumiscene Ultra are the easiest reagents to prepare due to activation tablet/s added to a ready made solution, this is not without some practical disadvantages. The shelf life of these reagents once the working solution has been prepared is displayed in table 5.1. This means that once the reagent has been prepared, then all of the solution must be used within the shelf life times or the solution is wasted. However, Bluestar Magnum contains three activation tablets so therefore a third of the solution can be used with one activation tablet making a total volume of 42ml each time. Lumiscene contains two activation tablets. Therefore half the amount solution may be used with one activation tablet making 250ml of the working solution. Lumiscene Ultra however contains one activation tablet so therefore all of the solution must be used making 250ml of the working solution. Grodsky luminol and Hemascein limit this potential wastage of solution because the user can prepare any volume depending on how much of the reagent is needed for a particular case.

In terms of applying the different reagent to a crime scene, Bluestar Magnum, Lumiscene, Lumiscene Ultra and Grodsky are all luminol based reagents. Therefore, they need darkness to view the reaction. Increasing the ambient light levels decreases the ability to see the chemiluminesence. A Hemascein reaction with blood, on the other hand, can be viewed when ambient light levels are not entirely dark. This makes movement around the crime scene easier. Also, photographing the scene is easier because the reaction can be viewed in relation to the crime scene without overlaying images as done in luminol photography (section 1.4.7). Also, because of the long reaction time of the Hemascein reaction, repeat

photography can be carried out. This is better than repeated applications of luminol as there is no further dilution of the sample.

Hemascein requires an external light source with a wavelength between 415-480nm to excite the fluorescein molecules. In the experiments in this thesis a polilight was used which was very bulky and needed a power supply. This could have some practical implications in some crime scenes, particularly crime scenes away from a power source and when attempting to examine a large area or follow a trail. However, there are UV lights available which are hand held and do not require and external power source. This would eliminate the problem with finding a power supply and carrying the external light source around the crime scene. The price of a UV light varies considerably but is an additional expense associated with using Hemascein as opposed to using luminol which does not require an external light source to view the reaction.

	Grodsky	Lumiscene	Lumiscene Ultra	Bluestar Magnum	Hemascein
Volume	500ml water	500ml solution	250ml solution	125ml solution	500ml water
Cost per kit (USD)	10.81	30-50	30-50	36	80
Cost per ml (USD)	0.02	0.06-0.1	0.12-0.2	0.29	0.16
Toxicity	Sodium perborate, Sodium Carbonate, Luminol	H_2O_2, Sodium Hydroxide, oxidising agents	H_2O_2, Sodium Hydroxide, oxidising agents	H_2O_2, Sodium Hydroxide, oxidising agents	H_2O_2, oxidising agents
Ease of preparation	Easy	Very Easy	Very Easy	Very Easy	Easy
Shelf life Unactivated	Dry powders-years	3yrs	3yrs	3yrs	7yrs
Self life Activated	4hrs	24hrs	4hrs	4hrs	7mths max

H2O2 Source	Sodium perborate 3.5g	2 H2O2 tablets	1 H2O2 tablet	3H2O2 tablets	1-3% H2O2 sprayed separately
H202 Content	~0.35%	0.12% max	0.12%max	Unspecified	1-3%*

Table 5.1: Summary of the economical and practical parameters for each reagent

5.1.5.6 Future Study

One study that would be useful in furthering the comparative investigation between the reagents in this thesis would be to investigate the effect of interfering substances. Interfering substances are explained in section 1.4.5.2. A question of interest is does increasing the sensitivity of luminol increase the chances of obtaining a false positive reaction? Also, it would be interesting to investigate what substances produce a false positive reaction from treatment with the different luminol formulations as well as treatment with fluorescein. Incidentally, interfering substances were indeed encountered in this thesis although not investigated. For example, in the sensitivity and longevity of reaction experiments the initial plan was to use white blotting paper as the substrate for the bloodstain. This was selected as it would limit the spread of blood and concentrate the blood more evenly than the cotton/polyester fabric which was later utilised. However, Lumiscene, Lumiscene Ultra and Bluestar Magnum all gave false positive reactions with the blotting paper while Hemascein and Grodsky did not. The fabric was then utilised which unfortunately reacted to a certain extent with Hemascein and Lumiscein Ultra, although not as drastically as the false positive reactions with the blotting paper. Interestingly, the least sensitive reagent, Grodsky, did not produce any false positive reactions with either of the substrates while the most sensitive reagent, Lumiscene Ultra, reacted to a certain extent with both.

Another study which would be beneficial to the comparison of these formulas would be to investigate the ability to obtain an mRNA profile from bloodstains treated with each of the reagents in this thesis. This was going to be investigated in this thesis however due to time restraints, this was not achieved. Section 1.4.4.4 describes the growing importance of mRNA in forensic science. An investigation into profiling

mRNA after luminol or fluorescein treatment is yet to be published and therefore this would make an interesting study.

Further investigation into DNA degradation and success of DNA profiling of luminol treated bloodstains would be needed for the reagents in this study. The 1:100 blood stained samples produced very small amounts of template DNA. DNA degradation was observed in the 1:100 bloodstain samples by comparison of the water treated samples with the reagent treated samples. Therefore, a question of interest is how much decrease in profiling success was due to low amounts of template DNA compared to DNA degradation? Also, the positive control for these experiments was water. Therefore, this only mimics crime scenes whereby water has been utilised to "clean up". Some cleaning agents are very alkaline which would alter the pH of the bloodstain prior to luminol delivery. Further study could incorporate the effect the different reagents have on DNA when bloodstains have first been washed by different types of cleaning agents.

Another study which would be of interest to forensic science would be the practicality and feasibility of employing the Matlab method, described in section 3.2.1, for detecting and enhancing very faint images of chemiluminescence. It was proven in this study that Matlab processed images allowed viewing of reactions that were either very faint or invisible to the unaided eye in unprocessed images making this type of enhancement valuable to forensic science. However, more investigation would be needed to assess whether this would work in real forensic cases. Hemascein images processed through Matlab were not representative of the bloodstain pattern due to background fluorescence. Investigation into the suitability of images of a Hemascein reaction processed via the Matlab method would need to be undertaken.

5.2 Application Method

This next section pertained to experiments evaluating the different application methods available for spraying luminol. The different spray types available in this

experiment were chosen to represent of the different types of sprayers available. Firstly, a pump sprayer was utilised as a representative of the type of sprayer most commonly used for the application of luminol by ESR during their crime scene examinations. The ABA spray was an example of an air pump sprayer and the ABA finger spray a representative of finger pump sprayers. The ECO spray was an example of a micro-sprayer with a disposable compressed gas reservoir. The Final spray type was a gravity fed spray gun connected to a compressed tank of nitrogen which represented the family of spray guns either connected to compressors or tanks of compressed nitrogen. These spray types are described in more detail in section 2.5.

These experiments primarily sought to investigate the ability of each sprayer to retain blood patterns on vertical and horizontal non-porous surfaces. Non-porous surfaces were chosen because this is the worst substrate type for retaining blood patterns sprayed with luminol. Tiles and vinyl was utilised as the substrates based on their porosity and because they are commonly found as substrates at crime scenes. Vertical surfaces showed if the application method prevented the luminol from "running" and horizontal surfaces provided a measureable spread or distortion to blood lines after luminol treatment. Grodksy luminol was used because it represents a common type of luminol used in forensic case work.

An additional experiment was included to attempt to explain the differences in results from the morphoanalytical study between the different spray types. This experiment sought to assess the spray density and size of the droplets expelled by each sprayer.

5.2.1 Spray Density and Size of the droplets

From the results of the experiment (section 4.4.1) and from figures 4.41, it was immediately apparent that the fine mesh holes of the pump sprayer, ABA sprayer and ABA finger spray nozzles and the air based systems of these sprayers were no match for the nebulised spray of the ECO spray and spray gun. The size of the droplets and the density of the spray mist were distinctly divided into these two groups. The pump sprayer, ABA sprayer and ABA finger sprayer sprays with a

mixture of droplet sizes and uneven coverage with some large areas of the graph paper void of the reagent when examined by application of the phenolphthalein solution. In contrast, the ECO spray and spray gun produced small droplets of a consistently small size which evenly covered nearly the entire sprayed surface.

For the first group, the pump sprayer performed the worst with droplet sizes significantly larger than the other spray types for the number of droplets sprayed in the given area. Figure 4.42 displays this relationship and table x displays the p-values related to this observation. The explanation of this observation could be due to either the size of the mesh holes in the nozzle of the sprayers or differences in spray pressure. Since some of the pump sprayer droplets are as small as both the ABA sprayers then pressure is probably the influential factor in this case. The pump sprayer works on a compressed air type system on a much larger scale. Therefore, pressure may be more readily dissipated out of the system during the spraying process. Reduced pressure would create larger droplets as this force is not strong enough to prevent combining of droplets after the liquid has left the sprayer in flight to the target. Also from figure 4.42 it can be observed that the ABA finger spray has a greater spread of droplet sizes than the other spray types. This may be due to the changes in pressure as the nozzle is depressed at different rates as opposed to the compression based systems of the ABA spray and pump sprayer which would deliver a more even pressurised force. The droplet density of these three sprayer types are similar with approximately 50% of the sprayed area covered with the solution, as shown in figure 4.44.

The ECO spray and spray gun produced finer droplets and coverd more area more evenly than the other sprayers because the liquid sprayed from the sprayer was finely dispersed in a vapour phase with the pressurised gas. The spray gun includes controls which control the amount of liquid and gas dispersed from the spray gun and width of the spray. These controls can be finely tuned to produce a nebulised spray mist finer than the ECO spray. The ECO spray includes no controls to adjust these parameters and the spray mist is nebulised at a constant force. Because of the constant force at which the gas nebulises the liquid, the droplets of liquid are

dispersed evenly over the surface of the target and covered the nearly 100% of the surface area.

Uneven distribution of larger sized droplets of luminol could interfere with the interpretation of blood patterns by mimicking spattered blood because the size of the droplets produced are in a similar range to spattered blood (section 4.4.1). Also, detail in blood patterns would be lost because the size of the droplets may be larger than the resolution of the pattern. Because the oxidised light emitting species of the luminol formula are not insoluble, the whole droplet would illuminate light regardless if part of the droplet did not cover the bloodstain or not. Sprayers which produce larger droplets are more susceptible to the "running" and "pooling" effect described in section 1.4.5.3 because more liquid is concentrated in a particular area for each droplet. If droplets are in close proximity to one another then the droplets have a higher tendency to "join" making the droplet even larger. If the droplet becomes large enough then the droplet will run under the influence of gravity or spread under the influence of surface tension. This effect ruins blood patterns as the light emitting species in the luminol formula are soluble in the liquid and will emit light following the path of the liquid. Therefore, the original locality of the bloodstain pattern is lost.

The density of the luminol sprayed onto a blood pattern is also important because light will only be emitted where the luminol has come in contact with the blood pattern. If areas of the blood pattern do not receive any luminol then loss of detail from the blood pattern may be observed.

5.2.2 Morphoanalytical Study

The observations and results of the morphoanalytical study were concurrent with the results of the spray density and size experiment. The larger droplet sizes produced by the pump sprayer, air pump and finger pump caused running and destruction to the vertical blood lines as seen in figure 4.46. However, the droplets produced by the ABA spray and ABA finger spray were small enough to resolve most of the 3mm blood lines and some of the 2mm lines though none of the 1mm lines. However, although the lines were resolved, running on some or the entire pattern was imminent. The ECO spray and spray gun produced droplets fine enough to out

perform the other spray types. The very fine mist produced by the spray gun enabled the 3mm blood lines to be resolved without the running effect.

For the horizontal blood lines, only the ECO spray and the spray gun had small enough droplets to reduce the amount of reagent spreading and meant it was possible to resolve the 1mm bloodlines. However, spreading was still observed as the luminol lines slightly wider than the original blood lines. The ECO spray luminol lines were significantly wider than the spray gun lines. The pump spray had droplet sizes which were too large and so spreading was observed which interfered with the resolution of all of the blood lines and destruction of these lines.

5.2.3 Practical and Economical Considerations

As with the type of reagent chosen for case work, the type of spray chosen for forensic case work is dependent on both the practical and economical factors as well as the performance of the spray. The factors considered in this thesis include the cost, ease of transport, ease of use, lifetime of the sprayer, the capacity of the sprayer and the practicality of employing the sprayer for forensic case work. A summary of these factors is displayed in table 5.2.

5.2.3.1 Cost

The pump sprayer was the least expensive of all the spray types having a wide price range depending on where the pump sprayer is purchased. Many manufactures produce the pump sprayer and can be purchased from many different places. The price of the ABA finger spray was undefined though is probably relatively inexpensive. The ABA spray had a single unit price of 15 USD. The ECO spray, including a compressed gas canister is 60 USD. Any additional gas canister cost 22 USD. The spray gun was the most expensive of all the spray types with the additional cost of the compressor or compressed nitrogen tank.

5.2.3.2 Ease of transport

All of the spray types in this thesis are easily transportable except for the spray gun because these spray types are all single, self contained and reasonably sized units. Transportation of the spray gun to a crime scene involves the transportation of

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either a compressor or a compressed nitrogen tank. Both the compressor and nitrogen tank are bulky and heavy making transportation more difficult.

5.2.3.3 Ease of use and practicality at a crime scene

All of the sprayers excluding the spray gun are very easy to use at a crime scene. The sprayers are filled with luminol and can be sprayed directly onto an area suspected of harbouring blood. The practicality of a certain sprayer depends on the intention of the investigator. For spraying large areas of a crime scene for the purposes of determining whether blood is present, the pump spray is more efficient because the capacity of the sprayer is more and the spray width is wider. This makes detecting of blood in this situation faster than the other sprays which cannot reserve as much luminol. However, if smaller regions of a crime scene or certain items need to be examined then the other sprayers may be more efficient. The smaller spray width and finer spray of the other spray methods reduce the amount of luminol used. This means that a single batch of luminol can be sprayed over a greater surface area, essentially reducing expense. This effect would be significantly beneficial if a more expensive but more sensitive reagent such a Lumiscene Ultra was used at a crime scene instead of the less expensive Grodsky formula. If the Lumiscene Ultra was utilised by a spray method which could reduce the amount of excess luminol used and still deliver enough luminol to a target for a bright chemiluminescent reaction, then despite the greater expense, the more sensitive reagent could be chosen.

The spray gun is not as practical to use as the other spray methods. If the spray gun is connected to a compressor, then a power outlet for the compressor must be available. Also the compressor is not as portable as the other spray types. If the spray gun is connected to a compressed nitrogen cylinder then again portability issues arise as the nitrogen tank is rather bulky and heavy.

5.2.3.3 Life time of the sprayer

The life time of the pump sprayer depends on the amount of use the sprayer gets. The lifetime of the ABA spray and ABA finger spray was not defined. However, both sprayers are disposable yet they were used multiple times during this study with no

apparent decrease in the performance of the sprayers. The ECO spray requires a gas canister which can be replaced at a cost of 22 USD. The lifetime of the spray gun and compressor is until broken. However, if the spray gun is attached to a nitrogen cylinder, then the nitrogen cylinder will need refilling.

	Cost	Ease of transport	Ease of use	Life time	Capacity
Pump Sprayer	Inexpensive	Easy	Easy	Until broken	5L
ABA Spray	$15 USD (ABA)	Easy	Easy	Disposable but can be used more than once	200mL
ABA finger	- (ABA)	Easy	Easy	Disposable but can be used more than once	5mL
ECO Spray	$60 USD (Bluestar)	Easy	Easy	Gas canister needs replacing ($22 US)	250mL
Spray gun	Expensive (gun+ either compressor or nitrogen tank	Moderate to difficult	Moderate, but need to find a power point if using a compressor and have to carry compressor/nitrogen tank around crime scene	Nitrogen tank needs replacing	100mL

Table 5.2: Summary of the economical and practical parameters for each spray type

5.2.4 Future Study

Future studies on the effectiveness of the different sprays in this thesis could include set up of experiments mimicking a forensic crime scene. For example, blood patterns could be made with shoes and fingers or impact spatter simulated at different blood dilutions. These patterns could then be sprayed with each of the sprayers and the amount of detail retained by comparison with each of the reagents could be observed. Also, a variety of substrate types could also be investigated from porous to non-porous surfaces.

5.3 Fixatives/Sheer Thinning Agents

This last section of experiments sought to solve the problem of blood pattern destruction by applying fixatives or sheer thinning agents. Fixatives and sheer thinning agents are discussed in detail in section 1.5. The ideal fixative should be able to not suppress the chemiluminescence of the reaction, preserve DNA integrity, not interfere with subsequent presumptive and confirmatory tests, be non-hazardous, inexpensive, chemically stable, sprayed simultaneously with luminol and preserve the morphology, position and detail of blood patterns.

Reduction in chemiluminescence could be due to the dilution of blood if the fixative has to be applied to the sample separately from the luminol. Spraying a fixative separately may be important to allow time for the fixative to fix the blood. Also, reduced chemiluminescence could arise from certain chemical properties of the fixative which may quench the reaction. A reduction in chemiluminescence essentially would reduce the sensitivity of luminol as the emitted light, already dim at low blood dilutions would appear even dimmer or even non-existent. Reduction in chemiluminescence could also come from chemical species in the fixative which may quench the reaction. One known quencher of cheiluminescence is acid (100). Luminol requires an alkaline environment for the conversion of luminol to its monoanionic and dianionic forms (section 1.3.4). These forms are prevalent at a pH range of 8-14. Depending on the acidity of the fixative, it could reduce the total pH of the luminol solution reducing this conversion. This would result in fewer reacting molecules of luminol which would reduce the overall chemiluminescence of the reaction.

Chemical stability of the fixative is important also. The fixative should react only with the blood and not with the luminol or the substrate on which the bloodstain is deposited or any environment factors that may be present. Therefore, the fixative

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must be able to withstand the alkaline environment in which luminol operates and not react with any of the chemical components of the luminol formula or the products and intermediates formed during the reaction of luminol with blood.

If the fixative could be sprayed simultaneously with luminol this would be more beneficial than having to spray the fixative onto the target surface separately. Apart from the above comments concerning further dilution of bloodstains, if a fixative is to be sprayed first then the area should be defined before the fixative is sprayed. Luminol is sometimes used to locate latent blood, therefore the blood stains may be invisible to the naked eye and it may be not known where to spray the fixative. However, in some cases, the general whereabouts of where bloodstains are suspected to be may be known. In these circumstances the fixative could be sprayed first. Another disadvantage to having the fixative and luminol sprayed separately is time. Extra time would be needed to apply the fixative and wait sufficient time for the fixative to dry or fix the blood before the luminol could be applied.

The most important attribute of a fixative for luminol, would be its ability to preserve the morphology, detail and position of a bloodstain. This would be achieved by preventing the oxidised products produced from the reaction of luminol with blood from diffusing away from the actual position of the blood. These species are water soluble and the luminol formula is predominantly water. Therefore as the water in the luminol formula flows away from the pattern due to gravity or general spreading, so will the oxidised luminol products.

The theory behind using a fixative is that it could essentially fix the oxidised products in place so that the luminol molecule reacting with these oxidised products will *in situ* emit chemiluminescent light. Therefore, theoretically only areas were the original bloodstain was will produce chemiluminescent light upon treatment with luminol.

The theory behind the sheer thinning agents was that thickening the luminol formula would help retain the light emitting oxidised molecules to the locality of the bloodstain due to inhibiting the flow of liquid. If the movement of the fluid is impaired then the movement of the light emitting molecules will also be inhibited. Therefore, the light emitting products, as with the fixative, should theoretically be restrained to areas where the original bloodstain was.

The fixatives used in this study were an alcohol fixative, two zinc based fixatives and two xanthan gum based fixatives. Descriptions of these can be found in section 3.6.3. A description of the mechanism of these reagents can be found in section 1.5.

5.3.1: Morphoanalytical Study

5.3.1.1 Vertical surface

All the fixatives and sheer thinning agents did reasonably well in this study except for the alcohol fixative. However, all of them out performed the control which consisted of the same blood pattern but with no fixative added. This control, when sprayed with luminol for 3 seconds retained none of the original blood patterns. This is because the amount of water present in the luminol solution carried away the reacting, light emitting luminol species due to gravity (figure 4.45). The alcohol fixative performed slightly better than the control, though the running effect was still observed. Interesting, the running effect was still present in the two zinc fixatives even though the lines, up until 1mm for Z1 and all of the lines for Z2, were resolved. Also apparent is a kind of "background chemiluminescent" effect which does not interfere with the resolution of the lines but decreases the contrast between the lines and the spaces between the lines.

An explanation for this effect is that because the bulk of the fixative is water, when the fixative is applied to the bloodstain before the luminol, some of the blood stain may dissolve into the water. The bulk of the bloodstain is, however, fixed. When the sample is dry, it may contain parts of the blood interspersed between the lines. When luminol is then sprayed onto the sample, the greatest chemiluminescence is

seen where the original blood lines were. The weak chemiluminescence between the lines could be a result of luminol reacting with the interspersed parts of blood between the lines. This effect could perhaps be reduced if less fixative was initially applied. However, reducing the amount of fixative delivered to a bloodstain may reduce the amount of blood which is fixed which would compromise the purpose of the fixative.

The running effect observed in the alcohol, Z1 and Z2 fixative samples could be a result of excess blood which did not fix, reacting with the luminol. This could create "free" oxidising agents which could react with luminol creating light emitting species showing the path of the water rather than the blood lines.

The xanthan gum and ABA fix sheer thinning agents gave very different results from the fixatives. Both of the sheer thinning agents were combined with the luminol formula before the luminol was sprayed which in itself is very beneficial. Having to spray only once reduces time and also allows does not require the investigator to have any prior knowledge as to where the blood stains may be. There was no running or background chemiluminesence effect with the samples because the amount of liquid is restricted. However, focal spreading of the chemilumnescent light away from the main blood pattern can be seen more frequently in the sheer thinning agent treated blood lines than the fixative treated lines. This focal spreading, seen prominently in figure 4.45, is perhaps where the solution has not been thick enough to keep the luminol from slightly running.

The ABA fix appears to define the blood lines better than the xanthan gum. The ABA fix is xanthan gum based but it is specially formulated for use with Hemascein. From these results it appears that ABA fix can also be utilised with luminol.

Lastly, a combination of the Z2 formula and ABA fix was used with Grodsky luminol. The ECO spray and ABA spray were utilised with this combination and compared and contrasted. The Z2, ABA fix, ECO spray combination performed better than the ABA spray/Z2/ABA fix combination and better than both the Z2 and ABA fix alone. With this combination, the background chemiluminescence effect of just the zinc

fixed samples, although still visible, was less pronounced and the running effect seen in the zinc fixed samples was non-existent. The lines of chemiluminescence were more defined as there were no blebs present as there was in the ABA fix treated samples. The explanation for this is probably that the xanthan gum prevents excess running due to the thickened luminol solution. Background chemiluminescence between the lines still occurs which is understandable because the zinc fixative causes slight displacement of the blood which is still reacts with the luminol. The background chemiluminescence may appear less bright because the lines are brighter due to the ABA fix keeping more of the luminol on the lines. The lines appear smoother and more to their original shape than they would with just the ABA fix alone due to the fixing capabilities of the zinc fixative. These features can be observed in figures 4.45.

The ABA spray/Z2/ABA fix combination managed to prevent the running effect and reduce the effect of the background chemiluminescence effect. However, because of the bigger droplet size expelled from the ABA spray and because they are thickened up with xanthan gum, the lines do not truly keep their shape. For the same reason, the chemiluminescence is not even over the image but there are bright and dull patches giving the image a speckled appearance. These features can be observed in figure 4.45.

5.3.1.2 Horizontal surface

Similar observations were found with the horizontal surfaced samples as with the vertical samples. No lines were resolved in the samples with no fixative. Three seconds of luminol application from the spray gun created pooling of the luminol and overall destruction of the blood pattern. Again the fixatives produced the characteristic background chemiluminescence as seen in the vertical samples while no background chemiluminescence was observed in the xanthan gum samples. The xanthan gum samples however did show the same bleb and speckled effect as was observed in the vertical samples. The Z2/ABA fix/ ECO spray combination again appeared to perform the best based on the images. This combination again combined the smooth fixing of the zinc fixative, creating well defined lines of fairly even chemiluminescence, with the thickened luminol formula preventing excessive

spread of chemiluminescence from the small amount of blood which might not have fixed. These features can be observed in figures 4.48.

The extent of distortion to the original blood pattern was assessed by measuring the lines and spaces of each sample before and after treatment. In most cases the Z2/ABA fix/ECO spray combination overall had the smallest amount of difference between the measured blood lines before and after treatment. This meant that this combination was the best at reducing the spread of luminol sprayed onto blood patterns. The next best reagents were the Z2/ABA fix/ ABA spray combination and the Z2 fixative. This combination did not perform as well as the Z2/ABA fix/ECO spray combination probably because the ABA spray expels droplets of greater size and unevenness than the ECO spray, as seen in section 4.4. The next best reagents were the Z1 fixative and the alcohol fixative. The Z1 fixative contained zinc acetate while the Z2 fixative contained zinc trifluoroacetate. This suggests that zinc trifluoroacetate is a better fixing agent than zinc acetate. Zinc trifluoroacetate contains more acetate groups than zinc acetate for denaturing proteins via salt formation or changes in pH (2). This the only difference between the fixatives, as the same concentrations of the protein cross-linking zinc chloride and the protein denaturing calcium acetate are used in both. The alcohol fixative is also a protein denaturing fixative though is not as effective at fixing blood as the zinc trifluoroacetate fixative. The xanthan gum and ABA fix were effective at stopping the luminol from running and pooling excessively, however, the thickness of the luminol formula caused an increase in the size of the droplets expelled by the spray gun. Also, because of the size of the droplets, the spray gun was prone to clogging. This resulted in uneven distribution of the luminol with areas of bright and dull chemiluminescence. Also, the droplet size interfered with the shape of the lines which made defining the edge of the line difficult.

Other fixatives were preliminarily assessed during this project but were not included in this thesis due to poor blood pattern preservation results. These included hair spray, which is essentially another alcohol fixative; polyethylene oxide, which is a sheer thinning agent; and various combinations of the various constituents of the zinc fixatives and the alcohol fixative used in this thesis. The polyethylene oxide

reagent was of a molecular weight of 10^6. The problem with this reagent is that it formed clumps in the luminol preventing the luminol from being sprayed. Perhaps a different molecular weight would be more efficient or otherwise a different method of combining the polyethylene oxide with the luminol, such as heating the luminol beforehand. The hairspray drastically decreased chemiluminesence intensity while preserving the blood patterns more poorly than the alcohol fixative eventually chosen for evaluation.

5.3.1.3 Future Study

Future studies on the effectiveness of the different fixatives/sheer thinning agents in this thesis could include set up of experiments mimicking a forensic crime scene. For example, blood patterns could be made with shoes and fingers or impact spatter simulated at different blood dilutions. These patterns could then be sprayed with each of the fixatives and the amount of detail retained by comparison with each of the reagents could be observed. Also, a variety of substrate types could also be investigated from porous to non-porous surfaces. Other fixatives and sheer thinning agents such as glyoxal and different molecular weights of polyethylene oxide could be investigated. The effect of DNA and RNA recovery after the use of different fixatives could be of importance. Finding a way to combine the zinc fixative with luminol would be very useful so that the two reagents do not have to be sprayed separately. Sensitivity experiments with the fixative combined could be important to investigate whether the different fixatives have a significant effect on the sensitivity of luminol or if this changes the interaction between luminol and the background producing false positive reactions. This would be of particular interest with the zinc fixatives because the addition of the zinc fixative to a blood pattern would dilute the blood to a certain extent. Different combinations of fixatives, sheer thinning agents, application methods and blood detecting reagents could be investigated to obtain a method which can preserve bloodstain patterns for forensic analysis.

6. Conclusion

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Luminol is an effective reagent for the detection and enhancement of latent blood and is widely utilised for this purpose throughout the forensic community. Luminol has many distinct advantages over other forensic blood detecting/enhancing reagents. Some of these include its superior sensitivity allowing luminol to detect very low concentrations of blood; the chemiluminescent light produced which can be recorded as evidence; and the efficiency in applying luminol to forensic case work.

However, luminol is not without some disadvantages. The emission of chemiluminescent light can be faint and requires a darkened environment which can often be impractical at crime scenes. The duration of the reaction is usually short making viewing, interpreting and photographing the reaction sometimes difficult. Like other chemical tests for blood, Luminol is not specific for blood and sometimes reacts with other substances in the environment give false positive results. Luminol may interfere with the profiling success of DNA and RNA. One major disadvantage of luminol is that the interpretation of bloodstain patterns is limited by the tendency of luminol to distort or destroy blood patterns, particularly on non-porous surfaces.

To overcome some of these disadvantages and to improve the attributes of luminol new formulas have been produced to improved results. The first section of this investigation sought to determine whether any of the new latent blood detecting formulas available at present were superior to a commonly used luminol formula, the Grodsky formula, and to determine which of these new formulas produced the best results. The new reagents evaluated in this thesis included Bluestar Magnum from Bluestar®, Lumiscence from Loci Forensic Products, and an improved version of Lumiscene called Lumiscene Ultra. In addition, I investigated an alternative to luminol, Hemascein. Hemascein is a fluorescein based blood detecting reagent. To investigate the suitability of these reagents for forensic uses, a set of experimental parameters for comparison were investigated. These parameters were sensitivity to blood, longevity of reaction, effect on DNA profiling and the ability to preserve blood

patterns on non-porous surfaces. Additionally, some non-experimental parameters were also investigated. These included the reagents toxicity, ease of preparation, shelf life, practicality for case work and the cost of the reagents

The second section of this thesis investigated the method of applying luminol by evaluating different sprayer types. The effectiveness of each sprayer was judged experimentally by the droplet size and distribution of the spray pattern in a given area and the effectiveness of the sprayer to preserve blood patterns on non-porous surfaces. Additionally, a set of non-experimental parameters were also investigated. These included the ease of transporting the sprayer to a crime scene, the practicality of utilising the sprayer on case work, the lifetime of the sprayer and its cost.

Many sprayers are available for delivering luminol. Some of the sprayers claim to limit the destruction of blood stain patterns. In this thesis five different types of sprayers were critically evaluated to compare their effectiveness. The sprayers included in this study were a common pump sprayer, the ECO spray from Bluestar®, the ABA spray and ABA finger spray from Abacus Diagnostics® and a nitrogen powered spray gun.

The third section of the thesis investigated ways of reducing the destruction of bloodstain patterns caused by luminol by evaluating the ability of different fixatives and shear thinning agents on the ability to preserved blood patterns on non-porous surfaces. A commercial fixative does not exist specifically for luminol at present. Therefore selected fixatives which are utilised in histology were investigated. These included an alcohol fixative of methanol and acetone in a 30:70 ratio; two zinc fixatives, one containing zinc acetate, zinc chloride and calcium acetate and the other containing zinc trifluoroacetate, zince chloride and calcium acetate. Shear thinning agents are another way in which to reduce pattern destruction in bloodstains treated with luminol. Two retention aids were investigated in this thesis. One of these was a shear thinning agent designed for fluorescein by Abacus Diagnostics® and is xanthan gum based. The second type was non-commercial xanthan gum.

6.1 Reagent Type

Out of the five reagents assessed in this thesis, Lumiscene Ultra showed the highest intensity of emitted light for higher concentrations of blood. However, when blood was diluted to lower concentrations, this intensity was comparable to Bluestar Magnum, Lumiscene and Hemascein blood detecting reagents. Therefore, the sensitivity of these reagents was found to be similar and could not be significantly separated. All of the aforementioned reagents, however, had a greater sensitivity than the Grodsky formula.

In this study, light was still reliably detected from the Grodsky treated samples at blood concentrations of 1:5000, as seen with the unprocessed images, and 6:100,000 for the Matlab processed images. The Bluestar Magnum, Lumiscene, Lumiscene Ultra samples showed reliable results at blood concentrations of 6:100,000, as seen with the unprocessed images, and 2:100,000 for the Matlab processed samples. The Hemascein samples showed reliable results at blood concentrations of 6:100,000, as seen with the unprocessed images and 4:100,000 for the Matlab unaveraged samples.

The reaction length of Hemascein was far longer than any of the other reagents. However, background fluorescence can pose a problem when viewing and photographing the Hemascein reaction with blood. From this study, it is recommended that any photographs of a Hemascein treated sample be taken within the first ten minutes of the reaction. Also, it would be beneficial if samples are left for one or two minutes after the application of Hemascein to allow for fluorescence to develop. However, in this study, at the blood concentration chosen (1:1000) the Hemascein reaction could still be seen at the reaction endpoint which was 2.5 hours after application of the reagent

For the luminol based formulas, Lumiscene Ultra had a significantly longer reaction than the other luminol based formulas. Although the reaction of the Grodsky

formula was less intense than the Lumiscene Ultra reaction, it had a comparably long reaction. The Lumiscene and Bluestar Magnum reaction lengths, relative to the reaction lengths of Lumiscene Ultra and Grodsky were very short. The Lumiscene Ultra and Grodsky reactions could still be reliably seen up to 4.5 and 5 minutes, respectively, while the Bluestar Magnum and Lumiscene reactions could only be reliably seen up to 2.5 minutes. Matlab processing improved visualisation of the images greatly. The Lumiscene Ultra and Grodsky images could be reliably seen up to 10 and 15 minutes after application of the reagent, respectively, while the Bluestar Magnum and Lumiscene images could be reliably seen up to 5 and 4.5 minutes, respectively.

From the DNA results, full, or near full DNA profiles were obtained from the samples with blood dilutions of 1:10 for all of the different treatments. Also, none of the samples showed any inhibitors of the PCR reaction. The quantification results for the Grodsky treated samples yielded very little DNA which resulted in comparatively small peak heights in the DNA profile. This showed that DNA was being degraded to a certain extent but not enough to inhibit the ability to obtain a complete profile. Degradation at this dilution was found in all of the samples relative to the water control samples. The Hemascein samples showed the least DNA degradation in terms of preserving the longer base pair STRs while Lumiscene Ultra had significantly less longer base pair STRs represented. Bluestar Magnum, Lumiscene and Hemascein could not be significantly separated. At a blood concentration of 1:100, none of the treatments managed to produce full profiles. However, all of the samples yielded less than 0.8ng of template DNA available for profiling. All of the reagents displayed profiles which were more incomplete than the water control samples. The Lumiscene Ultra treated samples showed significantly more degradation than the water samples. These samples, including the Lumiscene and Grodsky samples all failed to type longer STRs. The Hemascein samples showed the least degradation out of the different reagent treatments.

Hemascein combined with ABA fix managed to preserve blood patterns more efficiently than the luminol based reagents. Only the Lumiscene Ultra formula showed promise in preserving blood patterns out of all the luminol based reagents.

The Grodsky formula proved to be the least expensive reagent out of the ones evaluated in this thesis. The Grodsky formula was 15 times less expensive than Bluestar Magnum per volume luminol solution, 8 times less expensive than Hemascien and 6-10 times less expensive than Lumiscene and Lumiscene Ultra relative to the volume of luminol that can be made from a single kit. Bluestar Magnum was the most expensive. Lumiscene Ultra, Lumiscene and Bluestar Magnum, being tablet based reagents, were easiest to prepare. However, disadvantages to this include having to prepare all of the reagent, or at least an amount proportional to the amount of tablets in the kit, each time the luminol is prepared. For example, three 40ml solutions of Bluestar Magnum can be made with one kit because the kit contains three tablets. The Lumiscene Ultra kit only contains one tablet. Therefore, the entire solution has to be prepared. With the Grodsky and Hemascein kits, any desired volume of reagent can be prepared.

Also, the Grodsky formula can be modified by the forensic investigator to suit the case work conditions. The shelf life of the active Hemascein reagent is much longer than all of the other reagents. If stored properly, the active reagent can be kept for up to seven months as opposed to hours as with the other reagents. However, fresh solutions of the other reagents can be made up relatively easily and the unactivated forms of the reagent can be kept for years. In terms of applying the reagents in case work, the only other equipment needed for the application of luminol to bloodstains is a sprayer. The Hemascein reagent requires two sprayers, an external light source capable of emitting light at a wavelength between 415-480nm and orange or deep yellow safety glasses to view the reaction. When photographing the Hemascein reaction, a orange or deep yellow filter must be placed over the lens.

The type of reagent chosen for case work depends on what is needed in the investigation. Grodsky luminol may be preferred over the other reagents for spraying large areas for the detection of blood because it is the least expensive reagent. Grodsky luminol may also be useful for following footprints made in blood of high concentration. That way, the inferior sensitivity of Grodsky luminol is not such an

important factor. Also, unlike the other reagents, the formula of Grodsky luminol can be changed to suit the needs of the investigator.

However, Grodsky may fail to detect blood at lower blood concentrations which can still be detected by the other reagents. At a concentration of 8:100,000 Grodsky luminol no reaction was seen with the unaided eye. In contrast, all of the other reagents produced visible reactions at concentrations lower than this value. Another implication of low sensitivity is that if all background light at a scene could not be eliminated, such as moonlight, then this could further impact on the use of Grodksy for detecting blood at low concentrations.

The other reagents could be useful for special cases as described below.

Hemascein is useful for crime scenes where the blacking out the crime scene is impractical. It is also useful for detecting lower dilutions of blood and when DNA is required to be profiled after the blood is located with the reagent. If a longer reaction is required for viewing and photographing the reaction, Hemascein produces the longest reaction of the reagents examined. The ABA fix incorporated in the Hemasein formula preserves bloodstain morphology to a certain extent. However, Hemascein is more expensive than Grodsky luminol and requires an external light source and relevant filters.

Lumiscene Ultra can also detect low concentrations of blood down to a 2:100,000 concentration. At high concentration of blood, Lumiscene produces the brightest reaction and thus is more easily seen. Lumiscene Ultra also preserves bloodstain morphology to a certain extent, though not to the extent of the Hemascein reagent. However, Lumiscene Ultra is more expensive than Grodsky luminol and causes the most degradation to DNA.

Lumiscene and Bluestar Magnum produced very similar results with dilute bloodstains. Bluestar Magnum is more expensive than Lumiscene but did not out perform Lumiscene in any of the experiments performed in this thesis, apart from slightly less DNA degradation.

Also evaluated in this thesis was the use of Matlab processed images to detect and enhance very faint images of chemiluminescence. Enhancing these images allowed for detection of lower dilutions than what could be seen with the unaided eye. Due to background fluorescence, however, the hemascein samples could not reliably be processed and enhanced via Matlab. More investigation is needed to assess whether this method of enhancement would work in real forensic cases.

6.2 Application Method

In terms of droplet size and spray density, the ECO spray and spray gun had the best performance. These two sprayer types released smaller droplets and covered a larger area, in terms of absolute coverage, than the other sprays. Nearly 100% of the area was covered by a fine mist. In a practical sense, these sprayers would use a smaller amount of luminol and more efficiently cover a bloodstain with luminol than the other sprayer types. This means that more expensive reagents could be used to detect the blood because waste would be kept to a minimum. Also, because less luminol is applied to the surface, running and pooling of the luminol reagent is minimised. This helps to retain the morphology of bloodstains. The ECO spray and the spray gun were the only sprayers to resolve the 1mm blood lines. The ECO spray and spray gun are important in crime scenes where the preservation and resolution of blood patterns is important.

Conversely, the other sprays typically had a larger spray droplet size and covered a smaller area. The pump sprayer, ABA spray and the ABA finger spray covered around 50% of the given area with larger droplets. Therefore, the preservation of bloodstain patterns is comparatively worse. The pump sprayer was significantly worse at preventing the destruction of blood patterns than the two ABA sprays. However, the pump sprayer is best at quickly covering a large area with luminol, is the least expensive of the spray types.

The choice of sprayer, like the choice of reagent, depends on the crime scene situation. If a large area needs to be covered in a relatively short space of time, only

the detection of blood is necessary and the interpretation of complex patterns of bloodstaining is not required, then the pump sprayer may be favoured. The ABA sprays preserve blood patterns better than the pump sprayer but would take more time to cover an area. The ABA finger sprayer produces a spray with a very small width and would require considerable time to apply the agent

The ECO spray and spray gun would provide significant advantages at crime scenes where the preservation and resolution of blood patterns is important. Both of these sprayer types are more expensive than the other sprayers. The ECO spray is less expensive than the spray gun and is more portable. However, the spray gun allows the investigator to control how much reagent is delivered and at what force.

6.3 Fixative/ Shear Thinning Agent

The different fixatives evaluated in this study did have a significant effect on the spatial preservation of blood patterns. Each fixative however had its own advantages and disadvantages. The zinc fixatives and the alcohol fixative produced more defined lines than the xanthan gum and ABA fix shear thinning agents. Therefore the zinc and alcohol fixatives gave a more representative description of the exact locality and morphology of the original bloodstain pattern. However, although the lines of chemiluminescence were observed to be clear and decipherable, running and pooling to a certain extent was still observed. This was caused by excess luminol reacting with parts of the blood which did not fix. Also, when the fixative is first sprayed onto a bloodstain, the liquid in the fixative causes some of the blood to be suspended in solution which spreads out beyond the area of the blood staining. When the fixative dries and luminol is applied, the blood which was suspended in the fixative solution reacts with the luminol leading to chemiluminesence in areas away from were the blood pattern was originally present. However, this light is much duller than the light from the fixed samples and the pattern can be resolved.

The fixative which was most consistently produced the best results at retaining blood pattern position and morphology was the Z2 fixative. This fixative contained

zinc trifluroacetate while the Z1 fixative contained zinc acetate. The Z1 fixative was similar in terms of blood pattern preservation to the alcohol fixative.

However, the xanthan gum and ABA fix can be applied in combination with the luminol as one spray rather than spraying the fixative separately, as was required with the zinc and alcohol fixatives. Spraying separately not only takes more time, as time must be taken to allow the fixative to fix the blood and to dry, but also further dilutes the blood sample. This may lead to decreased chemiluminescence and possibly a decreased chance of obtaining a DNA profile. Also, The ABA fix and xanthan gum also reduces the running or pooling effect and the background staining problems of the zinc and alcohol fixatives. The ABA fix performed better than the xanthan gum at preserving blood patterns.

A combination of the Z2 fixative with the ABA fix performed the best at preserving blood patterns. The zinc fixative helped to clearly define the exact position of the bloodstain and the ABA fix prevented excess luminol from running or pooling on the sample. Combining this combination with the ECO spray worked very well. The ECO spray was the best sprayer type out of the ones evaluated in this thesis for the delivery of xanthan gum. However, the use of the ABA spray with the above combination proved that a less expensive sprayer could also be utilised to preserve bloodstain morphology.

Further study is needed to investigate the effect the different fixatives/shear thinning agents have on DNA recovery, subsequent presumptive and confirmatory tests, the effect of using more blood of lower concentration and suitability for forensic case work.

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