Fluorescein as a Field-worthy Latent Bloodstain Detection System

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Abstract: Fluorescein was considered to be the reagent with the greatest potential in the detection of latent bloodstains in the California Criminalistics Institute study by Mucieri and Monk in 1991 [1]. The purpose of this study was to develop and improve the fluorescein technique into a practical field system for the detection of latent bloodstains.

Current Material Safety Data Sheets (MSDS) state fluorescein to be no more hazardous than luminol, presently in use by many investigative organizations. Also, fluorescein has a twenty year history in the medical field of ophthalmology and is Food and Drug Administration (FDA) approved for clinical application in retinal and choroidal angiography [2].

Unfortunately, unlike luminol's single reagent application, fluorescin requires an application of itself followed by hydrogen peroxide (H_2O_2). This double reagent application can be problematic, especially on vertical, non-porous surfaces, resulting in bloodstain pattern distortion due to reagent running. A commercial thickener was used to overcome this problem, affording crime scene photographers greater opportunity to document the bloodstain patterns as evidence.

Introduction

During the 1980s, the Environmental Protection Agency became stricter with respect to toxic and/or mutagenic reagents utilized in the workplace, including those used as blood enhancement techniques. Many reagents previously used in a cavalier manner have been placed under greater scrutiny, resulting in the enforcement of more stringent

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safety guidelines. Luminol, one of the most commonly used bloodstain detection reagents, has been erroneously listed as banned for use in California in some literature. The current MSDS lists luminol as "possible carcinogenic"; hence, no such ban is in place on this reagent at this time. Nevertheless, finding a safe, reliable alternative bloodstain enhancement technique could be very valuable.

The literature lists fluorescein as the reagent with the greatest promise to replace luminol [3]. Although fluorescein is also listed as "possible carcinogenic", it has been used in the clinical setting since the 1940s, and more recently has been the angiography diagnostic mainstay for vascular ophthalmic disorders requiring FDA approval for clinical use [2]. This clinical heritage and FDA sanction may put to rest the safety issue with respect to the exposure to fluorescein.

The application of the fluorescein technique should be limited to latent stains only. The two factors which most directly bear on the effective use of this technique are either the blood concentration which has been so diluted it can no longer be seen by any other means, and/or the surface on which the stain is located lacks adequate color contrast differential. If any neat bloodstains are found, adequate samples should be taken for further serological testing before the fluorescein technique is employed. Due to the high alkaline nature of the fluorescein technique, any humoral studies (ie; ABO, anti-human, secretor status) may not be possible afterwards.

The substrate surface texture upon which the stain is located also plays an important role. Porous versus non-porous surfaces, and vertical versus horizontal factors are important to the success of this technique, or can prove to be problematic as in the case of a non-porous substrate on a vertical surface. To alleviate this problem, a commercial thickener, Keltrol RD, or xanthan gum, an exocellular heteropolysaccharide produced via fermentation, was employed [4]. This was added to the fluorescein diluent which helped to slow the dispersion or distortion of the bloodstain pattern after the fluorescein was applied, preserving the bloodstain pattern for documentation. With the increased viscosity, it became necessary to utilize a power-sprayer to produce even and consistent coverage for the target area. Previously, a hand spray pump bottle was adequate for this, and still is for the application of hydrogen peroxide. However, sufficient pressure is not possible for the more viscous reagent application for the fluorescein. A Low Pressure. High Volume (LPHV) spray gun was attained

because of its capacity to cope with the higher viscosity and ability to deliver the reagent in a controlled, contained and detailed manner.

In its reduced (colorless) state, fluores*cin* has a very short shelf life. The literature calls for usage within 48 hours. However, even after 24 hours the change back to its oxidation (colored) state, fluores*cein* was sufficient to cause problems with background fluorescence [1, 3]. Even though it is possible to prepare fluorescein in the laboratory and transport it to the crime scene, the fresher the fluorescin reagent is the more effective it will be, hence the need for field preparation. For this technique to be truly field-worthy, a premeasured and aloquated kit format was adopted. This required a more simplified preparation procedure, which should be within the skill level of crime scene technicians. Minimizing the equipment and skill required to prepare the fluorescin reagent is critical to its success in the field. Again, it must be stressed that this technique is to be utilized only after all other tests of interest have been performed and its only application is on latent bloodstains.

Materials and Methods

I. Reagents and Equipment
- Fluorescein
- Hydrogen Peroxide (H_2O_2)
- NaOH
- Hot Plate / Stirrer
- Zinc Powder
- Glassware
- Keltrol RD

II. Delivery Devices
- Hand pump spray bottles
- LPHV power spray gun

III. Documentation Equipment
- Alternate light source, 450nm filter and wand
- 35mm SLR camera, orange barrier filter (Nikon #056)
- *(Note: it has been suggested that a yellow filter may allow greater visualization)*
- Video camcorder
- Tripods, etc.

IV. Personal Safety Equipment
Particle face mask
Orange eye goggles
Latex gloves and lab coat

Reagent Preparation

Fluorescein Reagent

1) A 10% sodium hydroxide solution (10.0 grams sodium hydroxide in 100ml deionized water) is prepared. A stock quantity of this solution may be kept on hand provided it is stored in a nalgene type container. Storage of strong bases in glass containers is not recommended.

2) 1.0 gram of fluorescein is measured and allowed to dissolve in 100ml of the 10% sodium hydroxide stock, using a 250ml Erlenmeyer flask with a stir bar to facilitate dissolving.

3) While the fluorescein is dissolving completely into solution, 10.0 grams of zinc powder is weighed.

4) The Erlenmeyer flask with the fluorescein/sodium hydroxide solution is placed on the hot plate/stirrer, then stirred and heated gently. The 10.0 grams of zinc powder are added to this solution, and heated and stirred to a gentle boil. (Note: the zinc will not dissolve.) This solution will lose most of its color at this time. The solution is allowed to cool and the zinc to settle.

5) The cooled solution is decanted carefully to exclude any of the undissolved zinc. A 1:20 dilution (1 part [50ml] of the decanted solution with 19 parts [950ml] deionized water/dissolved commercial thickener) is made and mixed thoroughly. This will be the fluores*cin* reagent solution.

6) When the commercial thickener is used, approximately 5.0 grams Keltrol RD per 1000ml DI water in the total volume of the fluorescin solution is adequate. However, the Keltrol RD dissolves slowly and should be rehydrated in advance. This may be stored frozen, then thawed and used in the fluorescin's diluent.

Hydrogen Peroxide Solution

1) A 10% hydrogen peroxide solution (1 part [100ml] 30% hydrogen peroxide with 2 parts [200ml] deionized water) is prepared. A stock solution of this may be kept in an opaque bottle and refrigerated.
2) At the time of use, 300ml is placed in the second spray bottle.

Application Procedure

1) Spray bottle #1 (the 1:20 fluorescin dilution) is used to spray the area of interest. The area is misted with a fine, uniform spray from a distance of 12"-18". Two applications are made in this fashion while taking care not to make the misting so heavy as to cause the reagent to run. A few seconds are allowed for the color to develop.
2) Spray bottle #2 (10% hydrogen peroxide solution) is used to apply a mist in the same manner described above.
3) In accordance with standard safety practices for using UV light, all personnel must wear safety goggles.
4) The area of interest is illuminated with near UV light (450nm).

Application of the fluorescin dilution (bottle #1) on the targeted area of bloodstain will develop a yellow colorization within a few seconds if blood is present. At this stage, the bloodstain will be apparent and some background colorization may be apparent. However, the application of the hydrogen peroxide dilution (bottle #2) will help to reduce the background and false positive reaction, thus clarifying the bloodstain pattern. Using a near UV light source the bloodstained areas will fluoresce when illuminated.

Quality Control

If possible, a strongly reactive control, a weakly reactive control and a negative control should be simultaneously performed during routine examination. Also, the substrate of the area of interest should be simulated or an actual portion of the area of interest, properly labeled, should be utilized.

Limitations and Precautions

The purpose of this technique is to enhance the appearance of latent bloodstain patterns. It will not differentiate the type, or source of the bloodstain, and can yield false positive results with certain substances (ie; Fe, Cu, or soil [bacterial contamination]).

Two separate spray applications are necessary for this technique. A light and even misting yields the most successful results. However, non-porous vertical surfaces are susceptible to reagent running, which can distort or destroy any bloodstain patterns, and use of the commercial thickener is recommended in all circumstances.

Two major safety precautions are necessary with this technique. First, 10% sodium hydroxide (strong base pH 13.5) is extremely caustic and dangerous to work with. Also, storage of strong bases in glass containers is not recommended. Second, UV light can be very hazardous therefore the proper precautions must be implemented and safety goggles must be employed.

Results

The goal of this effort will be to focus on the following areas:

1) To simplify the chemical procedure for reducing fluores*cein* to fluores*cin*.
2) To verify aspects of Monk's results with fluorescein respective to sensitivity, false positives (specificity) and establish optimum working dilutions.
3) To resolve the reagent running problem, hence expanding the documentation window.

By attaining the forementioned goals, it is hoped that the fluorescein technique will be appreciated as a truly field worthy and practical procedure remaining within the scope and skill levels of crime scene technicians and investigators, hence resulting in an improvement in documentation.

1) Simplification procedure of fluorescein to fluorescin

In previous literature, fluorescein (oxidized color state) was reduced to fluorescin (reduced colorless state) via heating the fluorescein (1.0 gram) in 10% NaOH (100 ml) with 10.0 grams zinc powder. This was originally accomplished by the use of a refluxing column so as not to boil away the solution. This task may be routine for chemists, but would not be conducive to field production. The elimination of the refluxing apparatus and only bringing the solution to a boil does not appear to have reduced the effectiveness of the resulting fluorescin solution. Once established, this adaptation was used as the standard technique utilized in all subsequent experiments, which is previously delineanated in "Materials and Methods".

To determine if any sensitivity would be lost due to the adoption of the simplification procedure, the following experiment was conducted. Utilizing a common serial blood dilution (ranging from 1:1000 to 1:105,000) two racks of test tubes (12x75 mm) were set up. Each tube received equal portions (approximately 30 microliters) of blood dilution, fluorescin (1:3) and H_2O_2 (10%). Rack A utilized fluorescin

Figure 1

"The Kit"

reduced via the refluxing apparatus, and rack B utilized fluorescin reduced via the adapted simplification technique. Negative controls (blanks) were used with each rack in which the blood dilution was substituted with deionized water. Both racks were illuminated with a Wood's lamp (long wave UV). A plus/minus system was utilized to grade each tube for fluorescence. Equal results were achieved with each blood dilution and blank, establishing that the sensitivity was positive at 1:105,000 for both reduction techniques.

2) Specificity (cross reactivity/false positives)

The purpose of this experiment was to determine what other common substances may react with fluorescin yielding false positive results. All of the tested items are typically found in residential settings and may be mistaken for blood by either coloration (red/brown), or reaction to the fluorescin reagent (fluorescence). Table 1 notes the reactions observed during this test.

Table 1

Stained Items	*Fluorescence* inherent	UV (450 nm)
Control blood	-	+
Saliva	-	-
Coffee	+	-
Tea	-	-
Grass stains	-	-
Soil*	-	-
Chocolate	±	-
Cola	+	-
Strawberry jelly	+	-
Ketchup	±	-
Beet juice	+	±
Horseradish	+	+
Cherry-strawberry juice	±	-
Cherry-cranberry juice	±	-

* Soil is listed as positive in the literature; however, this soil yielded a negative result. Soils may vary greatly from one location to another.

Also Tested		
Urine	-	±
Steel	-	-
Steel (with rust)	-	±
Aluminum	-	-
Copper	-	+

Work dilutions

Two separate experiments were conducted during this study to determine the optimum fluorescin dilution to use. The first experiment was similar to the procedure simplification experiment (equal portions in test tubes) of testing varying dilutions of fluorescin ranging from 1:3 to 1:150 against blood dilutions ranging from 1:6000 to 1:105,000, as noted in Table 2. Grading the reaction with a zero to four-plus system resulted in the following:

Table 2

Blood Dilutions x 1000:

6	12	15	24	36	48	75	96	105	Blank	Fluorescin Dilutions
4	4	3	3	2	1	1	±	±	0	1:3
4	3	3	2	2	1	1	±	±	0	1:50
2	2	1	1	1	±	±	0	0	0	1:100
1	1	±	0	0	0	0	0	0	0	1:150

The second experiment utilized varying blood dilutions absorbed into strips of blotter paper. Based on the results of the previous experiment, the fluorescin dilution range was narrowed to a scope of 1:3 to 1:50. Due to the different substrate, the blood dilution was changed to a scope of 1:10 to 1:24,000. These results are listed in Table 3. Due to the difficulty in subjectively judging the reaction of the fluorescein on this substrate, a simple plus/minus grading system was employed:

Table 3

Blood Dilutions:

1:1000	1500	2000	2500	3000	6000	12000	24000	Fluorescin Dilutions
+	+	+	+	+	+	+	±	1:3
+	+	+	+	+	+	±	-	1:10
+	+	+	+	+	±	-	-	1:20
+	+	+	+	+	±	-	-	1:30
+	+	+	+	+	±	-	-	1:40
+	+	+	+	±	-	-	-	1:50

3) Documentation Improvement

Because of the need to apply two reagents (fluorescin dilution/hydrogen peroxide), reagent running was cited as a significant shortcoming to this technique [1]. If this problem could be overcome, it would improve documentation for court presentation. A commercial thickener, such as that used in "guaranteed one-coat" house paints (Keltrol RD) was utilized [4].

For this product to be useful in this technique, three criteria must be met: 1) it must be able to tolerate the high pH of the fluorescin without detrimental effects to its thickening properties; 2) it must not cross-react with the fluorescin, or cause any detrimental effects on the fluorescin's properties; and, 3) it must be capable of being applied in an even and consistent manner which is viscous enough to prevent reagent running and establish bloodstain pattern stabilization long enough for documentation. All horizontal and vertical glass panes were similarly bloodstained. The before (Figure 2) and after depiction (Figure 3) demonstrates the problematic nature of double reagent systems (fluorescin/H_2O_2), on a non-porous substrate (glass panes). The two glass panes on the right in Figure 3 illustrate the improvement with the Keltrol RD additive.

Early on, the manufacturer (Kelco) assured me that pH should not be a problem, since its product was derived via fermentation and endured other rigorous demands in that process.

The cross-reactivity would be known immediately as soon as the Keltrol and fluorescin were mixed together, and a blood sensitivity

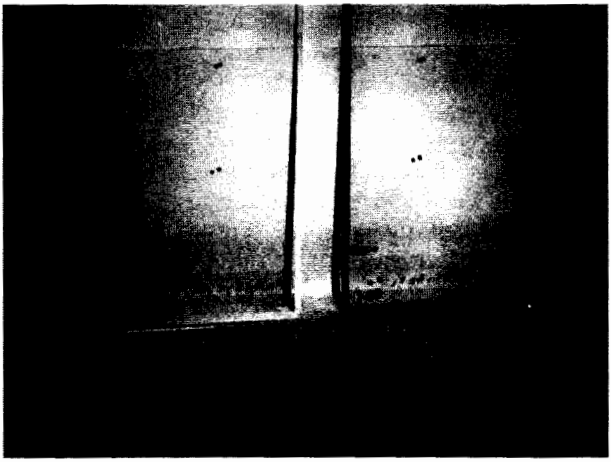

Figure 2

Glass panes with latent bloodstains

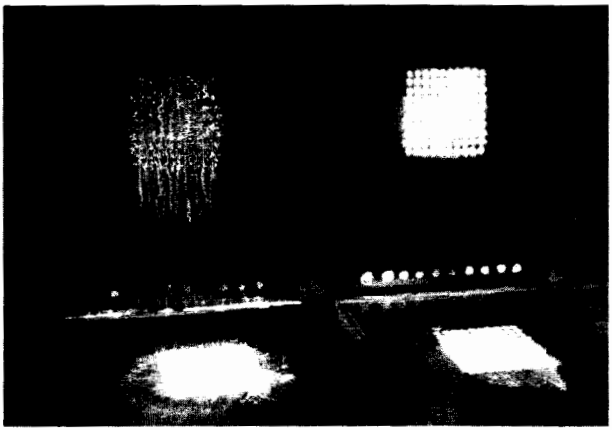

Figure 3

Same glass panes after processed with fluorescin without Keltrol RD (left) and with Keltrol RD (right)

gradient test was conducted without any negative side effect. In fact, the sensitivity was slightly greater with the Keltrol, probably due to stabilizing the reagents on the bloodstain, allowing the reagents more time to react. However, it was apparent immediately that the hand pump delivery system would not be capable of facilitating any viscosity necessary to alleviate the reagent running problem. Hence, a power spray delivery device would be necessary to satisfy the last (third) criterium. A 1.0% stock solution of Keltrol RD was utilized and later diluted to the desired viscosity (0.5%) and used in the fluorescin's diluent.

Keltrol RD Concentration	*Delivery System*
≤0.2%/volume	Hand pump spray bottle
0.3% - 0.6%/volume	Power spray device

Discussions and Conclusions

Considering that this technique would be one of the last procedures performed at a crime scene, it is possible for the crime scene investigators to notify the laboratory to prepare the fluorescin reagent and transport it to the crime scene. However, protection of the fluorescin reagent via darkened opaque nalgene bottles will be necessary. Exposure of the reduced (colorless state) fluorescin to sunlight or any UV light source may prematurely oxidize the fluorescin back to the oxidized (colored state) fluorescein, hence undermining the effectiveness of this technique.

The "Kit" concept yields greater latitude to the investigators with investigative organizations which have geographically large jurisdictions, and where transporting reagent from the laboratory to the crime scene is not practical for time or distance reasons. Also, the simplified reduction procedure requires less equipment and skill level to prepare, hence more personnel will be capable of performing this task, perhaps yielding better utilization of crime scene staff. Conversely, this technique can only be applied once to a target area. Each time a bloodstain is sprayed, to some degree dispersion of the pattern (distortion) will occur. Also, the background fluorescence will be very high on target areas which have been previously treated.

Once it was realized that a power spray device would be necessary to facilitate the increased viscosity of the Keltrol RD, a power spray

manufacturer was consulted. Originally, it was thought that an airless power spray would suffice because this type of sprayer would not emit the NaOH fumes except during the actual spraying. However the spray gun manufacturer advised the authors that only the most powerful guns could accommodate the necessary viscosity, and the high pressure (approximately 3000 psi) at which these guns operate would not be conducive to small areas or detailed application.

A Low Pressure (approximately 30 psi), High Volume (LPHV) gun was suggested, due to its ability to cope with the Keltrol's RD viscosity, and its ability to deliver the reagent in a confined and detailed manner, reducing the chance of overspray. Noxious NaOH fumes are emitted all the time the gun is powered, hence the need for the particle face mask. Due to the one-shot nature of this technique and the potential of destroying evidence, it is advised that only personnel with adequate training and experience should be permitted to employ this technique.

The documentational improvement afforded by this technique via Keltrol RD (xanthan gum), has further broadened the scope from investigative more toward evidentiary in nature [4]. This should result in better court presentation and ultimately a clearer understanding by the trier of fact. This may also broaden or expand the usage of this technique. Previously, this technique would not have been thought appropriate or effective in distinguishing class characteristics of shoeprints on various substrates, including carpeting. Visualizing a totally latent foot trail may lead the investigator to additional prints on a more favorable substrate, perhaps even yielding individualizing characteristics.

Preliminarily, results currently under study have demonstrated this technique to be at least as sensitive as luminol if not slightly better, although the literature empirically favors the sensitivity of luminol [5]. The effects of environmental conditions to which biological samples might be exposed by this technique, either from chemical or UV light, appear to be constrained and predictable according to current DNA analysis technology [6]. Preliminary DNA analysis via PCR has also been successful after employment of the fluorescin treatment. Both of these topics are slated for further study in the near future.

Documentation Guidelines

The fluorescin reaction can be documented with still photography or by video camcorder. The scene should be photographed prior to the fluorescin application. The substrate with the suspected bloodstain may then examined for any inherent fluorescence and any results should be documented by photography.

Prior to application, the fluorescin reagent should be tested on sample blood and checked for the proper reaction. The reagent should be applied on a like substrate, checked for cross-reactivity and the results documented. The reaction can be visualized and photographed with and without the use of an orange barrier filter. Unlike luminol photography, the scene does not need to be completely darkened for the reaction to be visualized and photographed. Some ambient light in the scene will aid the photographer/investigator in later orienting the scene and its contents in the resulting documentation, and alleviates the need for fill flash photography.

Photographic documentation of the fluorescin reaction is best accomplished with a tripod-stationed 35mm camera with the aperture set at *f*8 using an orange barrier filter and color print film (EI400). The exposure times should be varied bracketing between 5 and 30 seconds, depending upon the lighting conditions at the scene. Photographs may also be taken utilizing the aperture-priority automatic function of the camera, resulting in quality photographs.

The fluorescin reaction can occur for several minutes before the bloodstain pattern begins to degrade and background fluorescence becomes problematic. This allows ample time for the photographers to vary their exposures and document the scene with and without the orange barrier filter. (Note: a yellow barrier filter may also yield favorable results.)

The fluorescein latent bloodstain detection system has been adopted by the San Diego Sheriff's Regional Crime Laboratory, and is currently operational and employed when appropriate circumstances require such technique. It is the authors' hope that this study, and the improvements which it has produced, will add to the arsenal of scientific techniques necessary to discern the facts present at a crime scene.

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