Document Title:	Evaluation of a Novel Fluorescent Dye to Detect Ano-genital Injury in Women of Color
Author(s):	Kathryn Laughon
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Purpose of the project

Rape is an important public safety threat and defined as any completed or attempted, unwanted vaginal, oral, and/or anal penetration, committed by the use of force, threat of force, coercion, or victim incapacitation.. More than 20% of women and 1.6% of men during their lifetime experience a completed or attempted rape and more than half a million women (slightly over 1%) experience rape in a given year. Of these women, approximately 31% are non-white, including 10% Hispanic and 14% non-Hispanic Blacks (1). When sexually assaulted women report to the Emergency Department or sexual assault center for a medical forensic examination, an important aspect of the examination is documentation of injuries, including ano-genital injuries. DNA evidence can be important to establish intimate contact between the victim and offender but cannot establish consent. The presence of injuries, including ano-genital injuries, can sometimes provide evidence to corroborate the victim's history of events. These ano-genital injures can occur with any intercourse, consensual and non consensual, and are typically minor, occurring in the superficial dermal layers of the genitalia (2) which heal by regeneration, without scarring. Regeneration and mitigation can begin within 8 hours after wounding. Peak epithelial proliferation occurs within 24 to 72 hours after injury.

Injuries are defined as abrasions, lacerations, or bruises (3). This study focused on abrasions and tears. Abrasions are defined as superficial injuries to the skin caused by the application of blunt force (3). Tears or lacerations, are defined as ragged or irregular tears or splits in the skin, subcutaneous tissues or organs resulting from blunt trauma or trauma by impact (3).

Rates of genital injuries after sexual assault range from 32% (4) to 94% (5) with most studies reporting rates ranging from 40 to 60% (6). Even after consensual intercourse, however, genital injuries can be detected, though typically at lower rates than in cases when assault is reported. Rates of injuries after consensual intercourse range from 5% (7) to 61% (8) in studies in Europe and North American. The wide variation in rates of injuries likely reflects the lack of standardization in how injuries are visualized, types of injuries measured, and the populations included in study samples. These variations notwithstanding, across these studies the nonconsensual groups are more likely to have more than one injury (if injury is present) and more likely to have sustained abrasions and bruises (9, 10, 11). Careful documentation of the number, location, and type of injury is therefore important evidence that can corroborate (although not prove) a victim's claim to lack of consent.

Ano-genital tears and abrasions are best detected though the use of a stain to aid in their visualization. A 1% aqueous solution of toluidine blue (TB) stain is the standard (8, 11, 12). It is a general nuclear stain that highlights only nucleated cells. Because the normal surface of the vulva is not nucleated, there is not uptake of dye in uninjured areas (13). A growing number of researchers have noted that skin color is a factor in detecting ano-genital injuries with fewer injuries noted in darker skin. Several studies, using race as a proxy for skin color, have shown higher injury rates in white women compared to African American women after sexual assault (14, 15).

Given the importance of the use of toluidine blue in order to visualize any injury (for example, Zink and colleagues found 50% more injuries with the stain (2)), it is possible that the dark blue stain does not provide sufficient contrast to allow injury visualization in dark skinned

individuals. Additionally, in a summary report of a 2012 forum sponsored by the Office for Victims of Crime (OVC) and the National Institute of Justice (NIJ) to explore gaps in the existing research related to the technical aspects of sexual assault medical forensic examination, the problem of toluidine blue use on dark-skinned individuals was noted. Fluorescent stains are used in visualizing injury to other parts of the body, such as the cornea (16), but have not been used for this forensic purpose. A fluorescent stain would be visible regardless of the surrounding skin color through use of an alternative light source (ALS).

Topical fluorescein sodium in 1% and 2% solution is widely used in ophthalmology to visualize injuries and foreign bodies. The dye will enter spaces between cells, becoming more concentrated in areas where cell membranes have been disrupted or cell death has occurred (such as in injury) (17)

Fluorescein is considered to be generally safe. A search identified three case reports of serious events (anaphylaxis) noted with topical use of a 1% or 2% solution (18, 19, 20). A review of its safety for in vivo skin (intradermal) applications concluded that it "may be considered widely safe" (21). The vast majority of serious reactions associated with fluorescein sodium have occurred with intravenous or intrathecal use of the dye, when it is administered in far higher doses and in much greater concentrations (10% and greater) than when used for corneal staining or on the skin (17, 18, 21). Clinicians should always be alert to the possibility of an adverse reaction with the use of all substances.

In light of the above, we proposed to identify and test a fluorescent dye that will effectively stain genital injuries of skin color.

Aim 1: [Murine (mouse) trials] identify a suitable fluorescent dye and validate the dye in a murine skin injury model. Suitable dyes will be non-toxic, adhere to damaged epithelial (skin) cells and not to intact cells, be easily visualized using equipment already in common use among forensic examiners, and will not interfere with subsequent forensic DNA testing.

Aim 2: (Human trials) evaluate the safety, feasibility and efficacy of the identified fluorescent dye in women who have had consensual intercourse through a one-group trial. The dye will be used on the external genitalia of women within 48 hours after consensual penile vaginal intercourse to test application procedures, safety (local skin irritation and patient pain), and ability to detect genital tears and abrasions.

Murine trials

The procedures for the murine studies were described in detail (21). After obtaining permission from the Animal Care and Use Committee of the University of Virginia, a first study was conducted to evaluate whether or not the dyes adversely affected the rate of wound healing by applying them, alone or in combination, to similarly-sized linear abrasions (0.5-1 cm long) that were made in the dorsum of 5 mice. Wound lengths were evaluated 24 and 48 hours after wounding and dye application. By 24 hours, wounds in all four treatment groups (no dye, TB, FL, and TB+FL) were 50-60 percent shorter than they were at the initial time point; and wounds in all treatment groups were almost completely healed by 48 hours. In order to determine the clinical relevance of the wound healing, an equivalence test was performed to determine if the healing of wounds treated with FL was within +/- 20% of those treated with the standard dye, TB. There was a .01 cm difference in the means with a 90% CI (-0.186 ,0.166). We concluded that the two are clinically equivalent as the 90% CI was within preset range of -.2 to .2.

We then tested whether or not the dyes affected an observer's ability to accurately identify the wounds, blinded observers were asked to locate linear and irregular abrasions in photographs obtained from mice immediately after wound placement and dye treatment. Three blinded observers were able to accurately identify wounds ("true positive" identification) without dye 63.4+/-6.1% of the time, meaning that they failed to identify approximately 37% of the wounds that were present. When TB was applied to the wounds, the blinded observers were able to accurately identify the wounds approximately 98+/-1.3% of the time. Similarly, when FL was applied to the wounds, the blinded observers were able to accurately identify the wounds approximately 99+/-1% of the time, which was also significantly more accurate than without dye. The accuracy in correctly identifying wounds ("true positive" identification) was statistically similar between all dye and dye-combination groups. All single and combination dye treatments were significantly better than no dye for enabling the accurate identification of wounds ($p < 0.05$).

Our data support that when used at the doses evaluated here (1%), these chemicals do not significantly delay wound healing, supporting the safety of topical application of both TB and FL. However, there are some potential caveats of using FL in documenting rape cases that will require further evaluation before FL can be used clinically. First, this dye fluoresces in a wavelength (peak excitation at 494 nm and peak emission at 521 nm) that is currently used for DNA detection by the standard equipment used in most forensics laboratories. Therefore, if any FL inadvertently contaminates the DNA swab, its fluorescence might interfere with the fluorescence levels that indicate the amount of DNA in the sample.

Human Trials

A one-group feasibility trial was then undertaken with human subjects. Specifically, the researchers sought to determine if the dye was 1) safe (specifically was not associated with discomfort or localized irritation) and 2) effective at aiding in visualization of genital injuries. An additional aim was added later in the in the study to 3) determine if use of the dye in real-world application affected subsequent DNA profiling of vaginal swabs.

Methods: Approval for this study was obtained from the Institutional Review Board for Health Sciences Research of the University of Virginia. Women aged 18-45 who had engaged in vaginal intercourse with a male partner were recruited to undergo an external examination within 48 hours of intercourse. Eligible women had no serious health problems, were engaging in consensual intercourse with a man, were having periods regularly (or would be if they were not using birth control that suppressed menstruation) and were not pregnant. Interested women contacted the researcher, who explained the study, screened for eligibility, and provided a copy of the consent form through email. Interested participants contacted the researcher after having intercourse to schedule an examination. Neither race nor ethnicity was collected for the participants. The researchers instead used an objective measure of the skin color (described below).

At the beginning of the first study visit, women were asked the time and date of last intercourse and the examiner confirmed that the women experienced penile penetration. Examinations were conducted in a private examination room. A tristimulus colorimeter was used to document the color of their constitutive (untanned) skin. Skin color was measured using the Commission International d'Eclairage (CIE) L*a*b* (CIELAB) system where L* measures

lightness (white to black). These values can be associated with the values of the Fitzpatrick scale (22), a visual scale for assessing skin tone that has been used in previous studies of skin color and injury.

Their external genitalia were examined, and a photograph taken while the examiner used gentle traction to allow visualization of all of the structures. If any injuries were noted on gross examination, a close-up photograph was taken. A 1% solution of fluorescein sodium (compounded by the researchers) was placed on the external genitalia (labia minora, clitoral hood, clitoris, fossa navicularis and posterior fourchette) using a standard cotton scopette and then immediately removed with dry gauze. The room lights were dimmed and ultraviolet light (nm 540) was used to allow visualization of injuries stained with fluorescein sodium, if present. A photograph was taken. For certain study participants who consented four standard sterile cotton swabs were inserted into the vagina to collect a sample for DNA analysis. Once the participant was clothed, a buccal (mouth) swab was obtained for DNA analysis for those consented to DNA collection. A repeat visit approximately 48 hours later was scheduled for all participants. On study visit 2, the participant was again examined to determine if any irritation was noted and if injuries had healed (if injuries present on study visit 1). No dye was placed. Patients were also asked if they had experienced any discomfort (itching, burning or other irritation) since the study visit. Patients were called at a later date and asked if they had experienced any discomfort in the two weeks post exam. Participants were paid a total of $50 each.

Findings: 44 participants completed the study (one woman consented but then withdrew as she was not going to be available for follow up). Their mean age was 20.6 years (range 18.2-24.6,

SD 1.5). The mean time elapsed between intercourse and exam 1 was 22.1 hours (range 4.5-44.5, SD 11.1).   The L* values of their constitutive (untanned) skin, measured on the inner thigh, ranged from 47 to 69 with a mean value of 59.1 (SD 6.4).

Injuries were noted in 3 of the 44 participants. One abrasion was adjacent to the clitoral hood, and two participants had tears to the midline posterior fourchette. All injuries were visible both without and with fluorescein staining at the initial exam. For those with injuries at the initial exam, all injuries were healed at exam 2. No participant reported symptoms of irritation such as burning, itching or other discomfort at any time point. No signs of irritation were seen at examination two (48 hours) for any participants. On 14 day follow up, one participant reported a urinary tract infection diagnosed 13 days post examination. This was deemed an 'expected finding' for a sexual active women by the physician supervising the study.

Fluorescein sodium shows promise for use in identifying injuries in sexually assaulted women. This is a relatively young sample consistent with a college-age population. Their skin tones were predominantly in the medium range of Fitzpatrick categories (*L values for 54-82), but covered a wide range of skin tone from very light to dark. The injury rate of 7% was lower than that found in some other studies but within the range associated with consensual intercourse published by other researchers. This low rate of injury may have been due to the uniformly young age (mean of 20.6 years) of the participants. Other studies have included a wider range of age. There were no safety concerns noted for use of the dye for this application.

DNA testing

Tests to determine if use of fluorescein would potentially interfere with DNA testing of vaginal swabs inadvertently contaminated with dye were conducted in two phases. This testing was

conducted because a theoretical concern that the fluorescein may interact with the flurospectrometer to create false "peaks" that would alter the DNA profile in a manner that would render it not useful for forensic analysis. In order to conduct tests, five samples were collected from five human subjects (who did not participate in the later genital injury trials) in accordance with a protocol approved by the University of Virginia Institutional Review Board (IRB). Buccal cells were collected by vigorously rubbing the left and right cheek with two sterile cotton swabs held together. Each set of two swabs was placed in breathable cardboard carrying tubes. Samples from each individual were then placed in separate sealed envelopes.

The five samples from subject 1 were then divided into five treatment groups – unaltered, contaminated with 5 μl of 1%TB solution, contaminated with 5 μl of 1% FL in deionized water, contaminated with 5 μl of 05% FL in deionized water, and contaminated with 5 μl of 1% FL in deionized water and 5 μl of 1%TB solution. Enzyme-treated samples were first quantitated using a Nanodrop 3300 Fluorospectrometer prior to amplification using the Identifiler kit to target of approximately 1 ng total DNA They were then amplified in a BioRad MyCycler using the AmpFeSTR Identifiler PCR amplification kit according to the manufacturer's instructions. The amplified product was then separated and detected using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems,Carlshad, CA, USA). The raw data was analyzed using GeneScan analysis software. These analyses were conducted in the laboratory of James Landers and interpreted with the assistance of Dr. Landers and his research team. These analyses used equipment and procedures like those used by forensic laboratories for criminal justice purposes.

The initial testing showed that all the samples returned good matches to the control swab. The samples contaminated with FL, however, returned non-allelic peaks in the green and blue

ranges. These peaks appeared to be dose-dependent and reproducible. These artifacts result, we believe, from a detection limitation due to the inability of the detector to differentiate the fluor from the matrix standards, resulting in false, non-allelic peaks. In other words, the fluorescein dye created peaks that were not cause by DNA, but were artifacts.

Based on these findings, a second set of five sample swabs from three individuals were analyzed in the same manner. This time we wanted to ensure that the analysis artifact – the extra peaks – were reproducible and dose dependent. We therefore contaminated the swabs with 5 μl of 1%TB solution, 5 μl of 1% FL in deionized water, 5 μl of 0.1%FL in deionized water, and 5 μl of 0.01% FL in deionized water to compare to a control swab from each individual. These findings confirmed our hypotheses – the artifact, non-allelic peaks were reproducible and diminished with lower concentrations of the fluorescein.

We concluded that our preliminary findings suggest that fluorescein does not interfere with DNA typing. Because the peaks were reproducible, they could be excluded from the DNA profile.   We believe that the concentrations used in this small series were probably higher than the amount that might inadvertently contaminate swabs in the course of an actual examination, meaning that in real-world use the non-allelic peaks would be present very rarely. We therefore determined that it would be useful to collect vaginal samples from our research subjects to test potentially contaminated samples in a setting closer to real-world use.

In order to accomplish that aim, we then obtained IRB approval to collect vaginal samples from our research subjects while the genital injury study was on-going. Eight subjects consented. We obtained buccal swabs in the manner described above. We obtained vaginal swabs by vigorously rubbing the vaginal walls with 4 cotton swabs held together. The swabs

were then handled and analyzed as described above. As anticipated, the non-allelic peaks were not present in any of the samples, suggesting that that in real-world use, the fluorescein sodium does not contaminate samples at concentrations that interfere with female DNA profiles.

Products developed or in process

Bruce A, Cao Y, Henry C, Peirce SM, Laughon K. J. Forensic Sci. 2016. Pre-clinical assessment of safety and efficacy of fluorescent dye for detecting dermal abrasions in a murine model. Journal of Forensic Sciences. (e-pub ahead of print.)   doi: 10.1111/1556-4029.13173.

In process: manuscript entitled Safety and feasibility of a fluorescent dye for detecting genital injuries. For submission in winter 2017.

Implications for Criminal Justice Policy and Practice

Findings from multiple studies indicate that better evidence, in particular documentation of injuries sustained by the victim, can play an important role in the prosecutors' charging decision and subsequent successful prosecution of these serious, violent felony crimes. The findings from this study suggest that fluorescein is both safe and feasible to use in detecting genital injury across all skin tones, including dark skin. A 1% solution of FL visualized under blue light was equally as effective as TB in enabling the accurate identification of injuries by blinded observers in the murine studies and was effective for visualization of genital injuries in women after consensual intercourse in the human study, although the very small number of injuries precludes drawing a firm conclusion about the efficacy of the dye. FL did not delay wound healing in

either the murine or human study. It was not associated with any safety concerns in the human

study. Indeed, FL is currently widely used to visualize corneal perforations (15-16).

Implementation of this technique for injury documentation in the clinical exam room will

require, in addition to a single lens reflex camera, the availability of a blue light for illuminating

the wound and a yellow filter. Both of these items are widely available for less than $50 and we

do not anticipate these additional expenses being barriers to the use of FL in the clinical exam for

those forensic examiners already employing a camera that allows use of a filter (such as a digital

single lens reflex camera). Because fluorescein does not rely on contrast (unlike the current

standard dye, toluidine blue), skin color will not affect its efficacy. A larger sample and a

randomized control group will be needed to fully evaluate the efficacy of a fluorescent dye,

confirm that it does not interfere with DNA profiling, and allow for its use in criminal trials.

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