CUMULATIVE TECHNICAL PROGRESS REPORT FOR GRANT: 2000-IJ-CX-K013
TITLE: SpermPaint Optimization and Validation
REPORT PERIOD: June 30, 2003-December 31, 2006
REPORT DUE DATE: March 31, 2007
PRINCIPAL INVESTIGATOR: Dr. John Herr

Abstract

Monoclonal antibodies were developed to human proteins that were expressed post-meiotically during spermiogenesis and occupy specific domains in the sperm head and tail. The monoclonal antibodies were conjugated with fluorescent dyes and tested for their ability to identify sperm heads and tails in simulated sexual assault evidence eluted from swabs using fluorescent microscopy. Monoclonal antibodies to the acrosomal compartment head proteins SP-10 and equatorial segment protein [ESP] identified a cap shaped domain corresponding to the sperm acrosome and an equatorial band located in the middle of the sperm head, respectively. Monoclonal antibodies to the flagellar fibrous sheath component CABYR imaged the sperm flagellar principal piece. These reagents are predicted to be particularly useful in the rapid and conclusive identification of sperm heads and tails in sexual assault evidence, particularly in instances where sperm are few, where heads have separated from tails, or sperm components are masked by other cell types. Deployment of these immunoreagents will require a fluorescent microscope.

1. SPECIFIC AIMS

SPERMPAINT GOAL The identification of sperm remains a key component in microscopic analysis of sexual assault evidence and may be crucial to corroborating victim testimony. In instances where sperm are not readily observed, the forensic practitioner may search microscopic slides for several hours looking for a single intact sperm.

The goal of the SpermPaint Project was to develop monoclonal antibody probes to expedite identification of human sperm in sexual assault evidence. The project sought to develop protocols particularly useful for rapid identification of sperm when numbers of sperm are low in samples eluted from sexual assault swabs. Reduction in the amount of time required to positively identify human sperm in sexual assault samples is anticipated to provide a cost savings in forensic practice as well as expedite the number of cases processed, particularly in cases where sperm are mixed with a variety of other cells and unknown material.

Currently, the only stains available to aid in the identification of sperm are nuclear

and cytoplasmic stains [such as the Christmas Tree stain] which are not specific for sperm but stain a variety of cells including vaginal and cervical epithelial cells, bacteria and cells sloughed from the male accessory sex glands. This leaves the positive identification of sperm relying on discovery of the characteristic shape and form of intact sperm, which may prove difficult as the sperm head and tail separate very easily after the sperm are dried and eluted from swabs. Positive identification is particularly problematic where few numbers of sperm are present in the midst of a large number of other cells and debris. It was predicted that new antibody probes would allow a sperm flagellum or sperm head to be positively identified even if they are separated.

2. ANTICIPATED ADVANTAGES OF IMAGING BIOMARKERS UNIQUE TO SPERM

SpermPaint is anticipated to have several advantages for identifying sperm in forensic casework. The ESP and CABYR target proteins are unique to sperm and testis, being differentiation antigens that arise during spermiogenesis. This property of these biomarkers results in the fluorescent signal from both monoclonal antibodies being clear, bright and selective for sperm heads and tails with no cross reactivity to other tissues. The target antigens ESP and CABYR are stable. SpermPaint identified sperm in samples collected 1, 24 & 72 hours after coitus on swabs that were then stored at 4°C for more than two years.

Significantly, the SpermPaint reagent: 1) identified sperm heads and tails when they were detached from one another and the shape and form of the sperm under light microscopy were otherwise difficult to discern; 2) identified sperm heads and tails that were masked by adherence to vaginal epithelial cells, even when the epithelial cells covered the sperm; and 3) identified sperm hidden within cellular debris. These performance characteristics of SpermPaint are anticipated to allow more rapid identification of sperm in microscopic fields and increase the number of conclusive identifications.

3. THE SPERMPAINT REAGENT

The current formulation of SpermPaint is a mixture of fluorescent dye conjugated monoclonal antibodies that allows definitive identification of human sperm using fluorescence microscopy. The current formulation of SpermPaint contains: 1) a monoclonal antibody specific for the sperm head antigen, equatorial segment protein [ESP] and 2) a monoclonal antibody specific for the

sperm flagellar antigen, calcium binding tyrosine phosphorylation regulated protein [CABYR]. Together, this mixture of monoclonal antibodies stains the sperm head with a characteristic band pattern through its central region corresponding to the equatorial segment and stains the longest domain of the sperm tail corresponding to the principal segment. [See Figure 1]. The cocktail of two monoclonal antibodies: 3C6-AF to the human sperm acrosomal antigen ESP and 3A4-AF directed to the sperm flagellar antigen CABYR are labeled with fluorescent probes. ESP and CABYR monoclonal antibodies were labeled with AlexaFluor 488 fluorescent dye from Molecular Probes. AlexaFluor has absorption and emission wavelengths of 494nm and 519 nm, respectively. This emission wavelength can be observed with filters commonly used to observe FITC fluorophores.

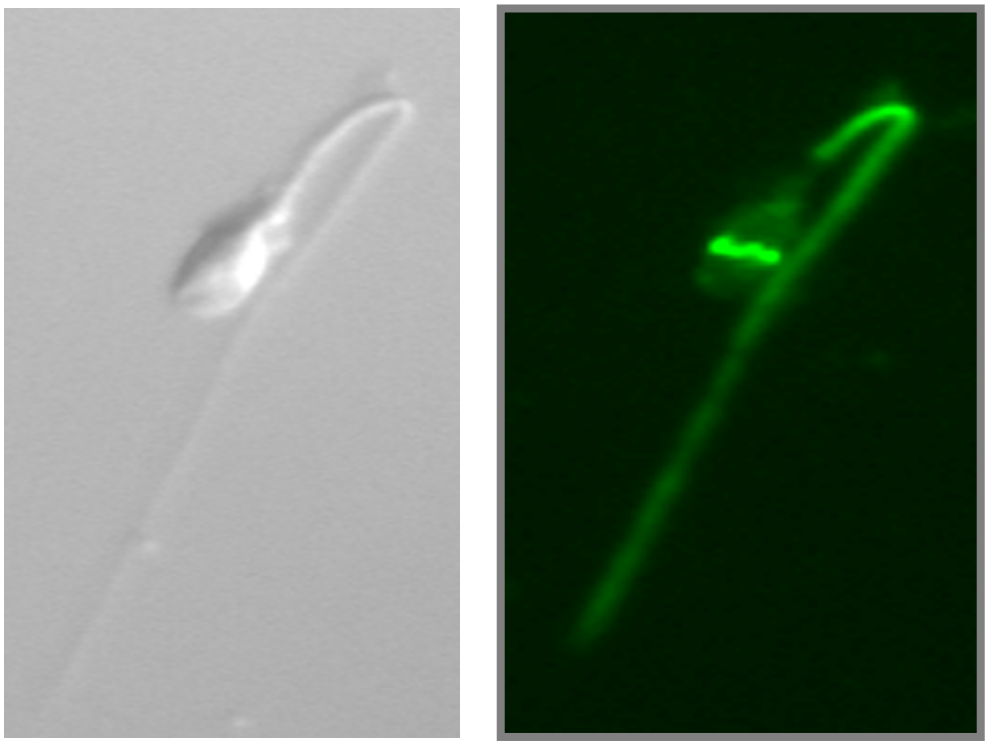


Fig. 1. The current SpermPaint formulation consists of monoclonal antibodies to ESP and CABYR. Equatorial segment protein [ESP] is localized to a discrete 4-5 um wide band in the middle of the human sperm head corresponding to the equatorial segment (10). The other protein, calcium binding tyrosine phosphorylated protein [CABYR], is localized to the fibrous sheath of the human sperm tail (9,11). Monoclonal antibody 3C6 to ESP and 3A4 to CABYR are thus able to localize specifically to the head and tail domains, respectively. Together they illuminate the sperm head and tail as can be observed above.

4 METHODS EMPLOYED IN THE IDENTIFICATION OF SPERM BIOMARKERS

4.1 Sperm Proteomics: High Resolution 2-Dimensional Gel Electrophoresis Was Employed to Identify Novel Sperm Proteins. The differentiation and development of the sperm involves the expression of unique genes and proteins, "differentiation antigens." The goal of the2D gel proteomics phase of the project was to identify the proteins unique to the sperm and evaluate their suitability as forensic targets.

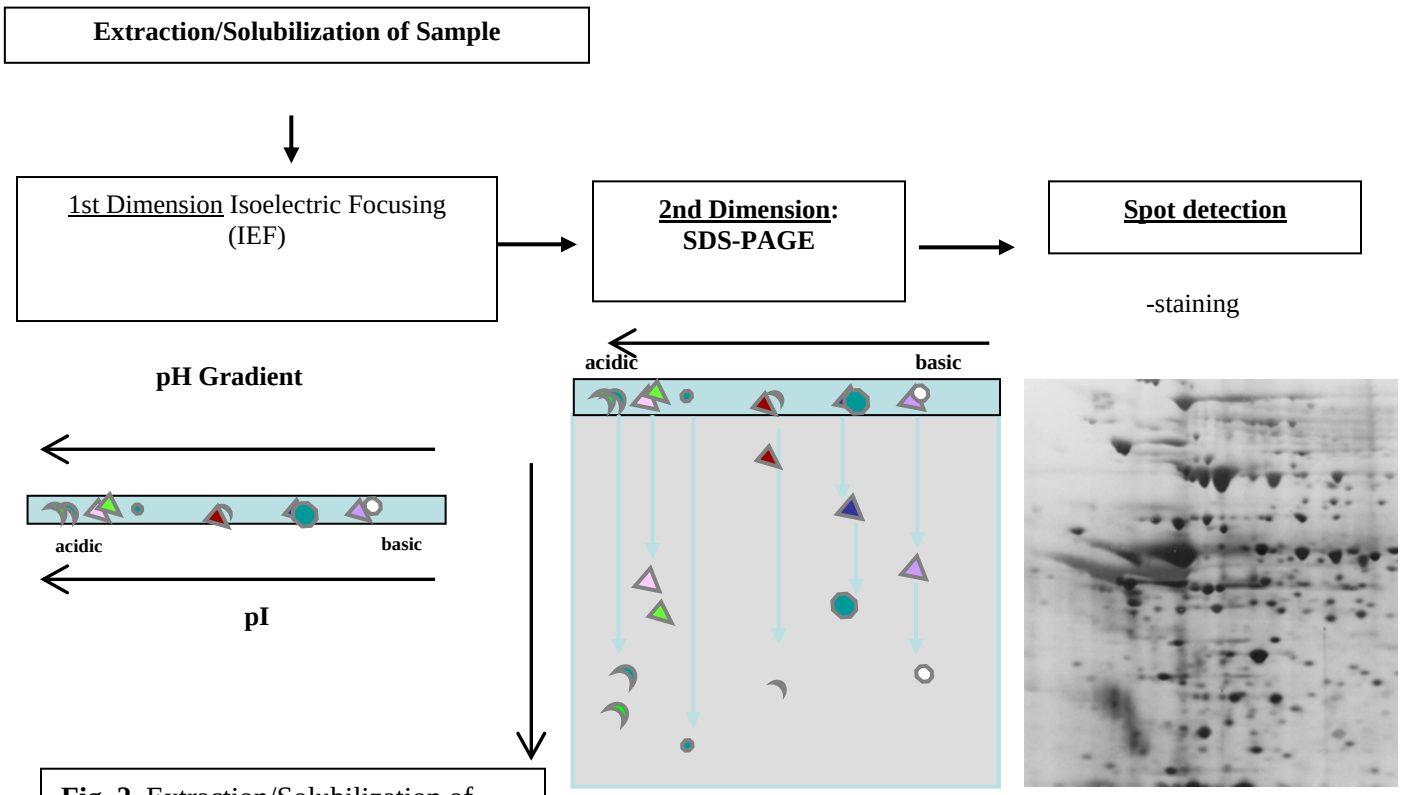


Fig. 2. Extraction/Solubilization of Sperm Samples and 2-D Electrophoresis.

4.2 Specific Discovery Criteria for Sexual Assault Target Antigens. Sperm proteins were analyzed according to several criteria to identify potential biomarkers:

1) *Accessibility*: Proteins localized to sperm domains predicted to be accessible to antibodies.
    - Sperm plasma membrane
    - Acrosomal membranes
    - Cytoplasm underlying cell membrane
2) *Processing Resistant*: Protein retained on sperm eluted from dried swabs by conventional procedures.
3) *Stability*: Protein epitope stable on evidence after years of storage.
4) Protein immunogenic in humans and/or animals.
5) Tissue specific, unique to sperm.
6) *Domain selective*: Specific to sperm head or to tail.
7) Unique Pattern: Characteristic features fostering rapid recognition by the forensic analyst.

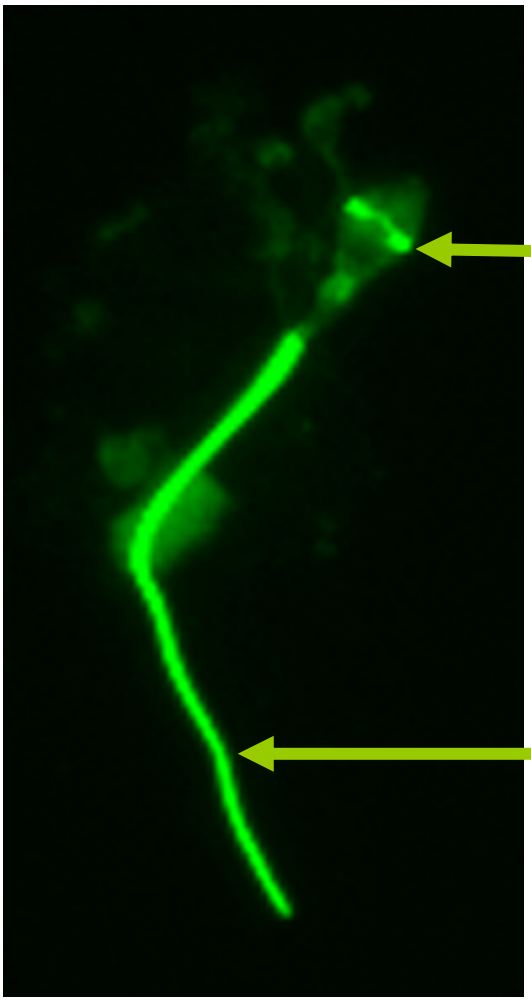


Fig. 3a. 3C6-AF mAb recognizes protein ESP and stains the equatorial segment of the sperm head.

Fig. 3b. 3a4-AF mAb recognizes protein CABYR and stains the principal piece of the sperm tail.

4.2.1 Why ESP Was Chosen as a Forensic Target on Sperm.

- ESP is Sperm-Specific
- ESP protein first appears following meiosis in round spermatids and persists on the acrosome of the sperm head within the equatorial segment. [Fulfills the criteria of a "differentiation antigen."]
- Because of its equatorial segment localization ESP gives a distinctive head banding pattern readily identified by the analyst.

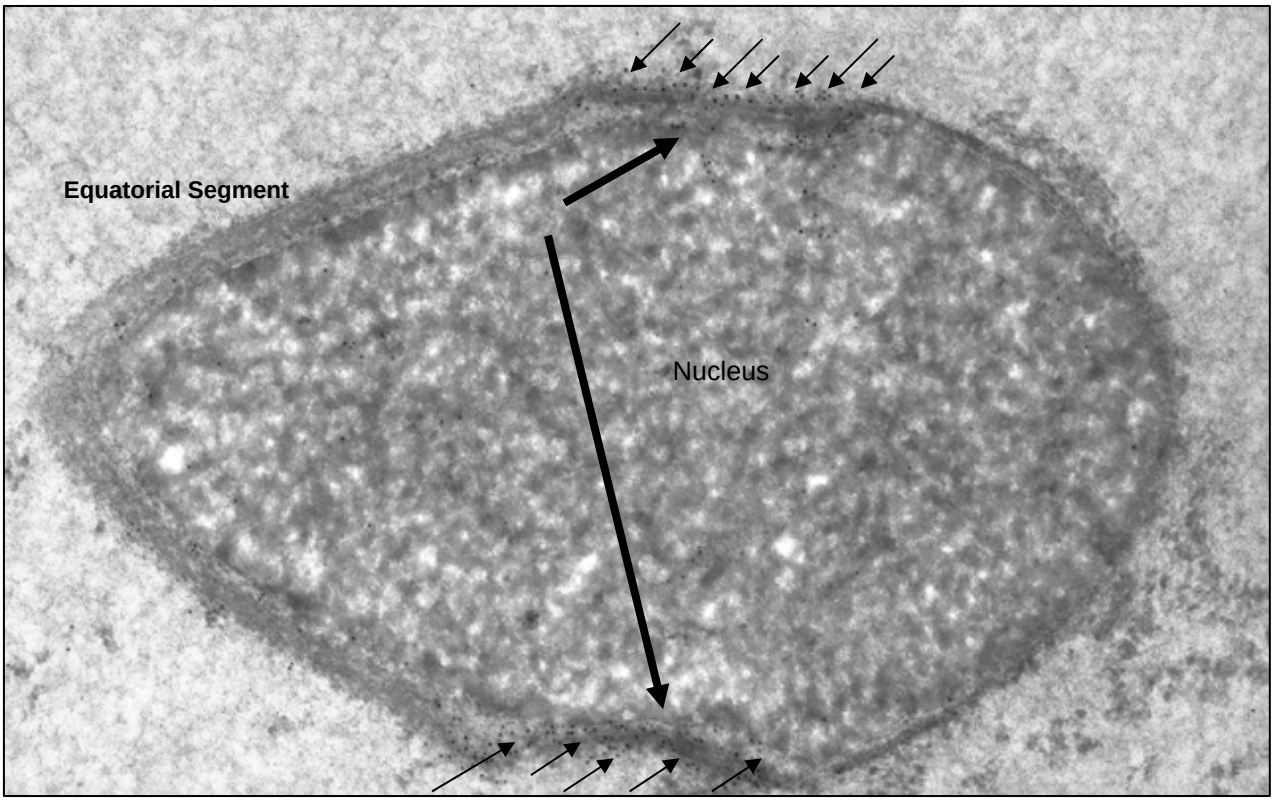


Fig. 4. Monoclonal antibody localizes ESP to the equatorial segment of the sperm head. Electron micrograph of a human spermatid in the testis. Sections were incubated with 3C6 monoclonal antibody, followed by gold conjugated secondary antibody. Gold particles indicate the abundance of ESP in the equatorial segment.

4.2.2 Why CABYR Was Chosen as a Forensic Target on Sperm.

- CABYR is Specific to Spermatids in the Human Testis
- CABYR protein first appears following meiosis in round spermatids and persists in the principal piece of the tail [Fulfills the criteria of a "differentiation antigen."]
- CABYR gives a distinctive tail banding pattern readily identified by the analyst.

The Forensic Sperm Target CABYR: <u>A Novel Calcium Binding Tyrosine-Phosphorylation Regulated Fibrous Sheath Protein</u>. CABYR was discovered by combining information on proteins phosphorylated during capacitation with methods to detect new calcium binding proteins. In Figure 5 below, immunofluorescent micrographs are shown of paraffin sections of human testis showing CABYR localization to human spermatids.

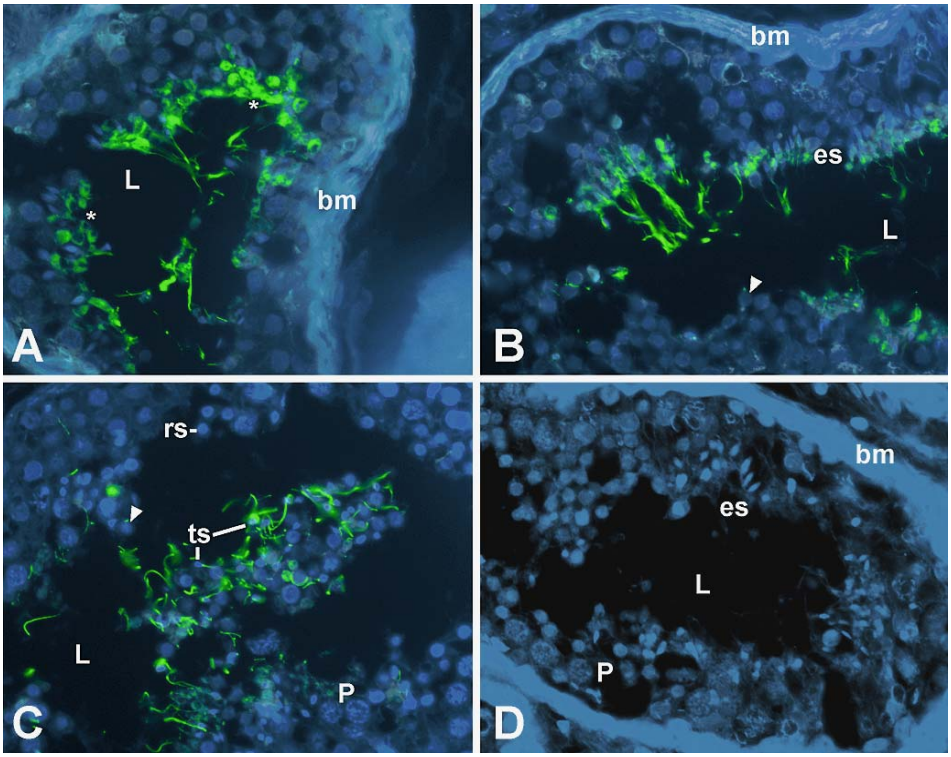


Figure 5. CABYR is localized to round (rs) and elongating spermatids (es) in the seminiferous epithelium and to testicular spermatozoa (ts) within the lumen. In round spermatids staining was observed as a small spot (arrowhead), possibly representing the flagellar organizing center (arrowheads in B and C). In some round cells, dense staining was observed throughout the cytoplasm (* in A). In early elongating spermatids staining was observed at the posterior pole of the cell as a short, thin strand. As the spermatids matured, staining appeared to broaden and lengthen as the tail lengthened (B, left side of image). In testicular spermatozoa (C) staining was associated with the sperm tail. No fluorescence was observed with preimmune serum in panel D. Abbreviations: bm, basement membrane; L, lumen; P, pachytene spermatocytes; rs, round spermatids; es, elongating spermatids; ts, testicular spermatozoa.

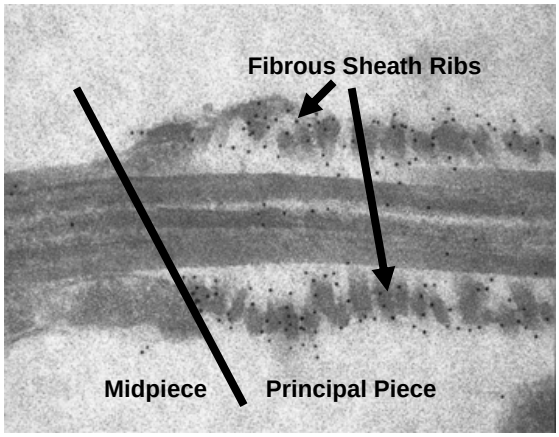
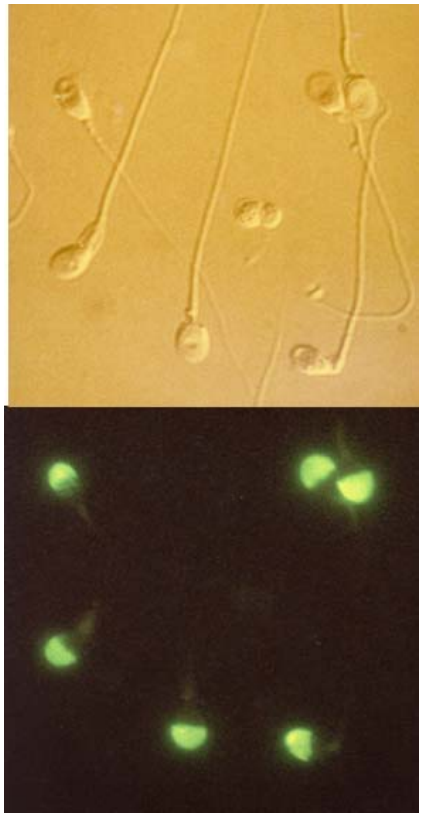


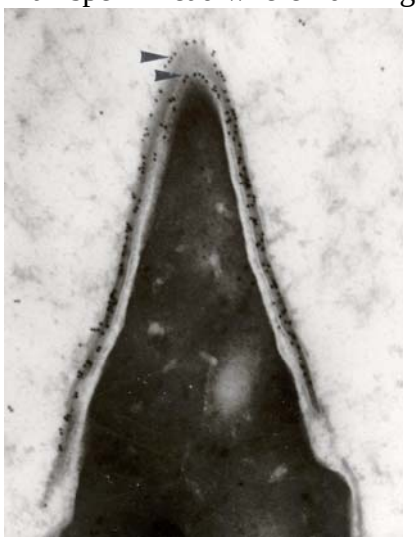
Fig. 6. **Monoclonal antibody to CABYR localized CABYR specifically to the fibrous sheath in the principal piece of the sperm tail.** In this electron micrograph of the junction between the midpiece and the principal piece of the sperm tail, black dots are 5 nm gold particles conjugated to antibody indicating the presence of the protein CABYR. No staining is evident in the midpiece or the axoneme.

SPERMPAINT PLUS

A monoclonal antibody, MHS-10, directed to the intra-acrosomal protein SP-10 was also developed. This monoclonal antibody stained the entire acrosomal cap of human sperm [see figure below]. Thus, the anterior two thirds of the human sperm head is illuminated by this monoclonal antibody probe.



In the panel to the left the monoclonal antibody MHS 10 reacts with the human sperm head and displays a cap shaped pattern of immunofluorescence. In the electron micrograph below the cap shaped localization pattern seen in the light microscope is revealed to correspond to the acrosomal compartment in the human sperm head where 10 nm gold particles coated with the monoclonal antibody lie within the acrosomal matrix.



A sperm-detection assay. We used the monoclonal antibodies to SP-10 antigen to develop a very sensitive assay for few numbers of sperm. This assay can detect as few as 200,000 sperm /ml.

Normal levels are 20,000,000-150,000,000 sperm /ml. For the purposes of developing a rapid assay the SP-10 antigen is better that the CABYR antigen or ESP antigen because CABYR is a cytoskeletal element and SP-10 is a globular protein much more abundant and soluble in the sperm head.

5. VALIDATION OF SPERMPAINT ON SIMULATED EVIDENCE.

Samples from post-coital donors collected on cotton-tipped swabs at various time points from one hour to 72 hours after sexual intercourse. These swabs were stored in cardboard containers at 4°C for over 2 years.

SpermPaint Protocol:

1. Sample swabs were rehydrated in 0.5ml PBS per swab for 20 minutes and then agitated manually at 5 minute intervals to release cells into PBS. 25 µl of PBS suspension was applied to microscope slide and air dried at room temperature overnight or on a slide warmer for 2-3 hours, not to exceed 37°C in order to preserve antigenic sites on the sperm.

2. Sample on slide was rehydrated by overlaying 50-100µl PBS directly onto sample area. At this point, the slide may be treated to fix the sperm.

3. The sample is fixed by applying 50- 100µl of 4% paraformaldehyde for 20 minutes. The 4% paraformaldehyde is aspirated and the sample is washed 2-3 times with PBS. If not fixing the slide, skip to step 4. Good results have been obtained without fixing the slide.

4. If the examiner chooses to fix the slide, the PBS is aspirated. SpermPaint is diluted 1:25-1:50 in PBS with 10% Normal goat serum and applied to sample on slide. (50µ-100µl diluted SpermPaint per slide). Keep slide incubated in a closed container with a wet paper towel on the bottom of container to prevent the slide from drying out. Best results are obtained with incubation overnight at 4°C. Good results are also obtained incubating for two hours at room temperature.

5. SpermPaint is aspirated and the sample is washed five times with PBS.

6. Anti-fade reagent is applied and the sample is covered with a coverslip and sealed with nail polish. Slides are stored flat in a covered folder and stored at 4°C until viewed.

5.1 EXAMPLES OF USEFUL APPLICATION OF SPERMPAINT ON SIMULATED EVIDENCE *NOTE: All following Illustrations are actual post-coital samples eluted from cotton swabs.*

Figure 7. SpermPaint can find that one sperm obscured by a whole field of epithelial cells.

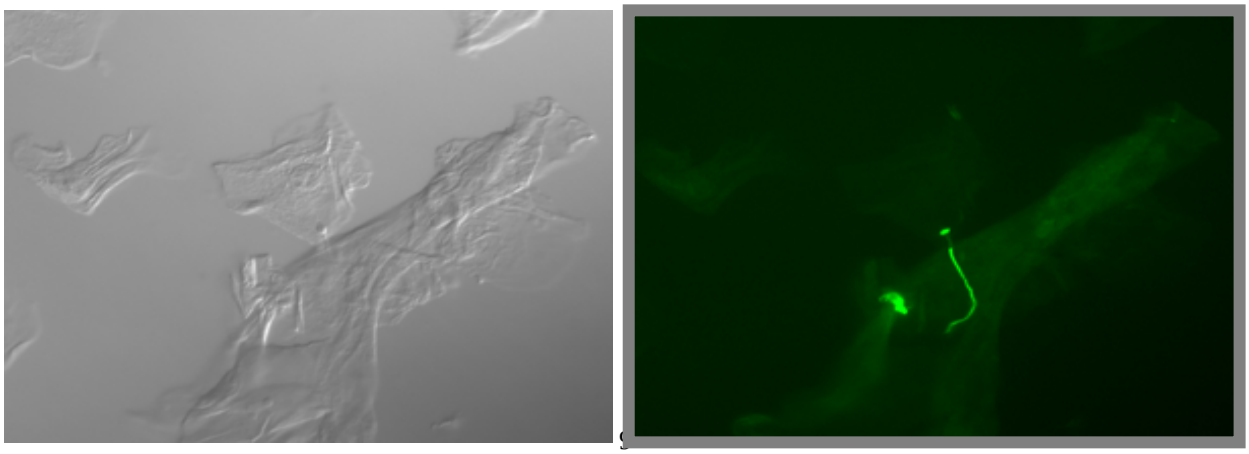


Figure 8. An otherwise questionable small fragment can be positively identified as sperm in origin, as seen in this sample recovered on a cotton swab 24 hours after intercourse.

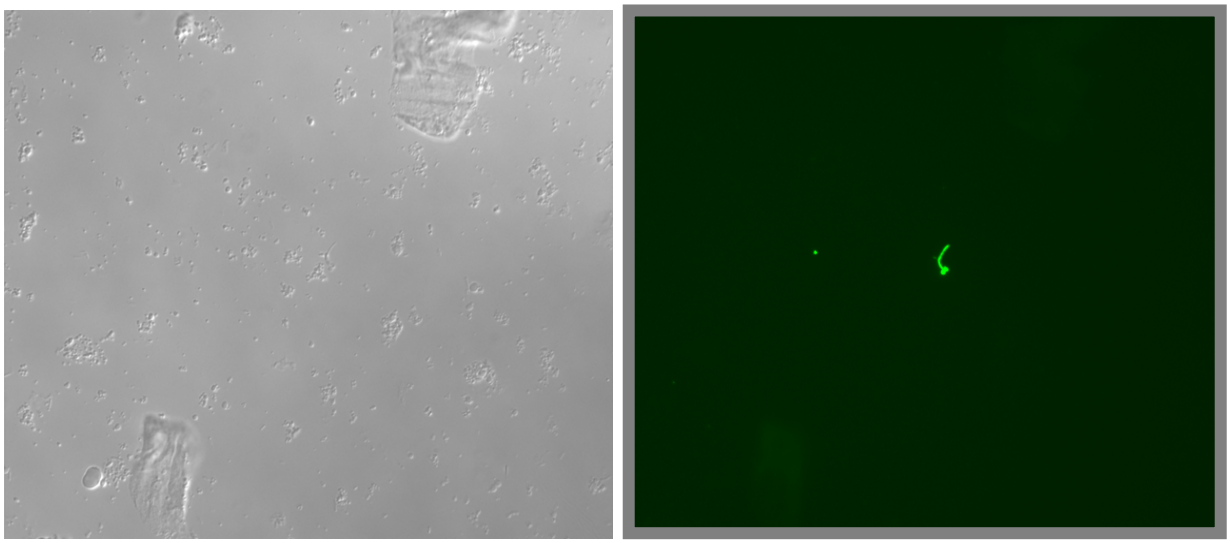


Figure 9. Sperm can be detected in samples collected on swabs 72 hours after intercourse and stored dessicated for over two years.

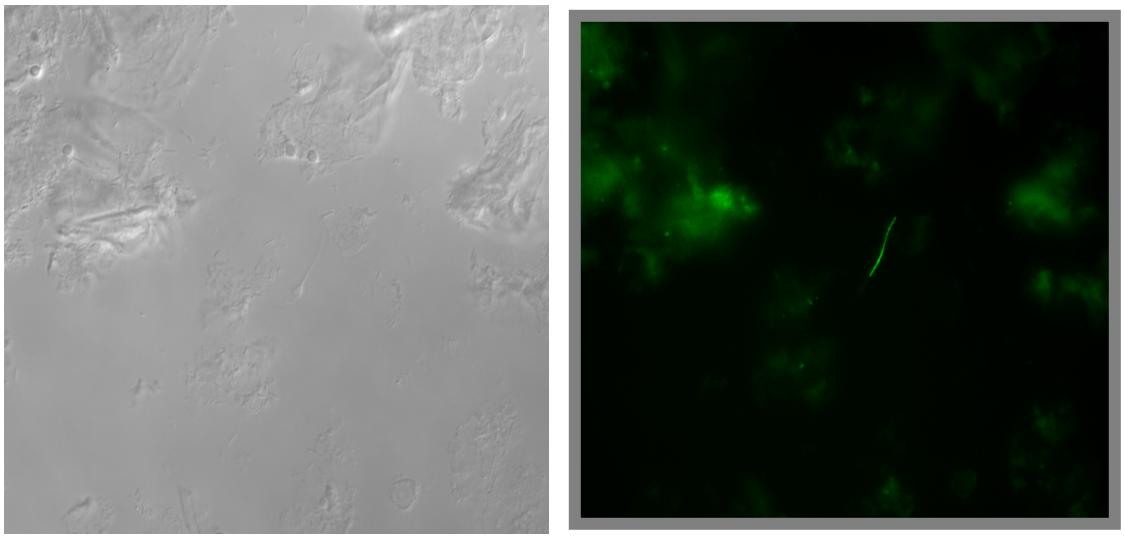


Figure 10. SpermPaint can identify sperm heads and tails that are most often separated from each other in sexual assault evidence recovered from swabs. The antigens detected are very stable when the samples are stored dessicated. Shown here is a sample collected one hour after intercouse. The slide was prepared from a swab that had been stored dry for over two years at 4°C; the slide was stored for an additional 7 months before staining.

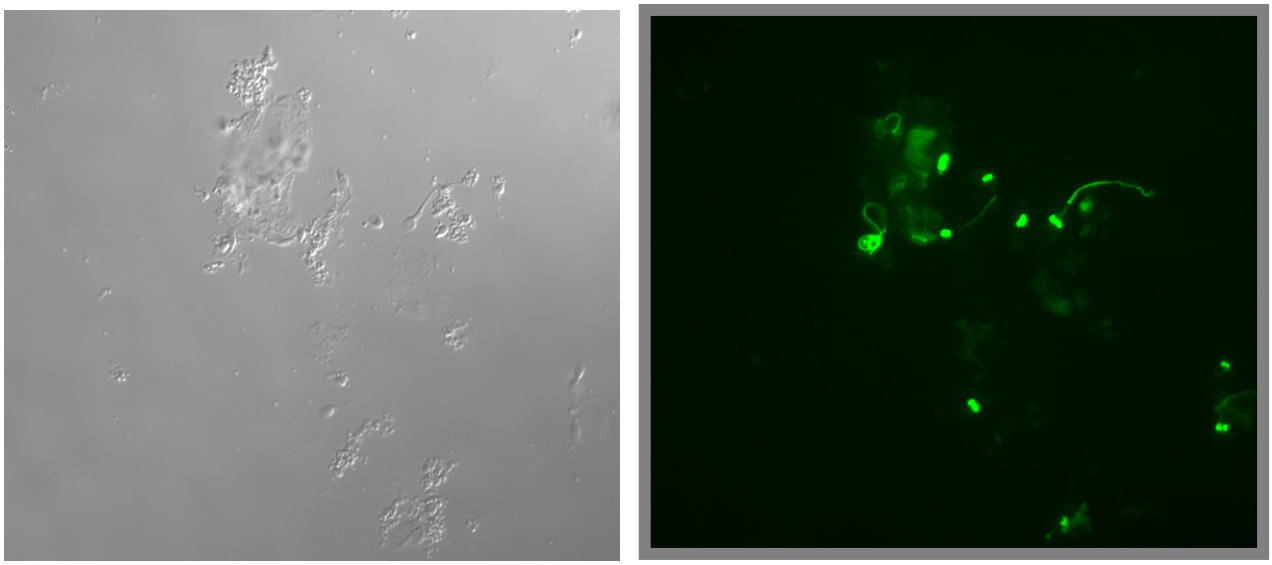
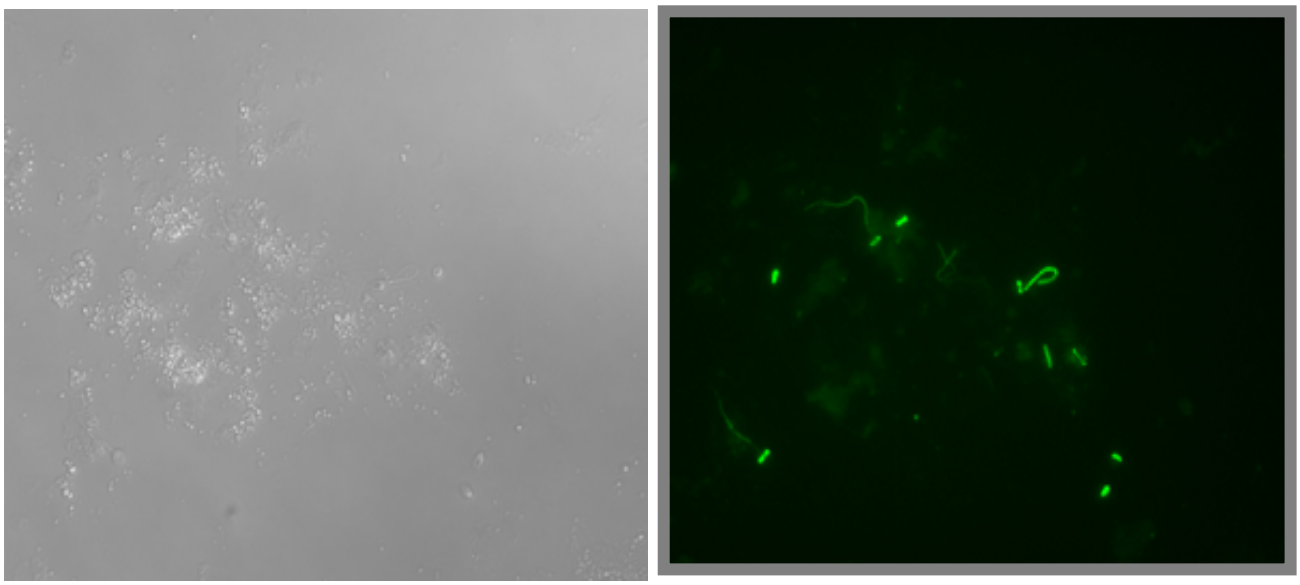


Fig. 11. SpermPaint can easily distinguish spermatozoa in a field full of debris.



5.2 Binding characteristics of SpermPaint on simulated evidence at various intervals post-coitus.

SUMMARY	Time Post-Coitus											
	1 hr	%	6 hr	%	12 hr	%	24 hr	%	48 hr	%	72 hr	%
Heads	71	13%	200	32%	37	16%	10	18%	0	0%	1	33%
Tails	165	29%	280	44%	117	52%	41	75%	6	86%	2	67%
Intact	328	58%	151	24%	71	32%	4	7%	1	14%	0	0%
Totals	564	100%	631	100%	225	100%	55	100%	7	100%	3	100%

The SpermPaint reagent was employed to identify numbers of intact sperm, isolated sperm heads or isolated flagella in smears eluted from swabs at various post-coital intervals**.** Data from four couples is summarized in the table above. The SpermPaint reagent recognized the ESP [head] and CABYR [tail] biomarker proteins up to 72 hours post-coitus, indicated that the epitopes recognized by the ESP and CABYR monoclonal antibodies were stable and persisted on sperm heads and tails through these intervals. Concerning the rate at which sperm undergo degradation: intact sperm were found in significant numbers at 1, 6 & 12 hrs [means of 58, 24, & 32%], respectively. At the 24-hour interval, fewer intact sperm were noted [5-13% intact, average 7%].

At 48 and 72 hour time points, sperm were more difficult to discover, while tails were identified most frequently. Beginning one hour post-coitus and extending through all subsequent time periods, the numbers of sperm tails identified by SpermPaints in samples from all couples predominated over sperm heads. This finding indicates that sperm flagella persist longer than sperm heads, particularly in the samples taken from later post-coital intervals.

Because the flagellum contains the midpiece of the sperm, where the mitochondria are located, the persistence of flagella in the longer post coital intervals suggests sperm flagella may be useful for mitochondrial DNA fingerprinting.

Identification of SpermPaint stained spermiophages in 48 hr post-coital smears. Amoeboid macrophages could be identified in post-coital swabs and smears. The macrophages were distinguished from squamous vaginal epithelial cells by their characteristic smooth surface projections, more hyaline cytoplasm and the fact that the squamous epithelial cells display sharply defined plasma membranes with sharp edges [expand the above micrograph to 200% to see these features]. There macrophages likely enter the vagina by leukocytosis in response to semen (2,3). Single macrophages [from 48hr post-coital smears] are centrally displayed in each phase contrast photomicrograph left (left images above) in fields surrounded by squamous vaginal epithelial cells. When viewed with fluorescence microscopy after staining with SpermPaints (right images), these macrophages contained immunofluorescent signals for CABYR and ESP antigens in their inclusion bodies, definitively identifying them as spermiophages. Key subjects for future study include: the incidence of spermiophages at various post-coital intervals and whether sperm DNA persists within spermiophages in a sufficiently intact form to permit DNA fingerprinting.

Preliminary Results on Minimal Sperm Numbers Required for mitDNA analysis. A pilot study was carried out at Mitotyping Technologies using low numbers of freshly collected sperm and subsequent mitochondrial DNA analysis. It was observed that 20 isolated sperm were sufficient to successfully amplify a 350 base pair PCR product in the majority of experiments. In contrast, 10 sperm gave results less than 50% of the time. These preliminary results indicate that collection of 20 sperm or sperm flagella offers a good starting point for amplifying mtDNA in the proposed experiments.

6. SUMMARY

Results Using SpermPaint. The fluorescent signal from both monoclonal antibodies is clear and bright with no cross reactivity to other tissue.

- SpermPaint has demonstrated that it is effective in identifying sperm in post-coital samples that were stored at 4°C for two years. Target proteins stable in 1, 24 & 72 hour samples.
- Positively identifies questionable shapes.
- Identifies sperm adherent to epithelial cells.
- Identifies sperm hidden within cellular debris.

Anticipated Benefits of SpermPaint. More sperm will be identified.

- Fluorescing sperm targets will be readily discerned in the field.
- Conclusively identify sperm heads and tails when they are detached.
- Conclusively identify sperm heads and tails when they are adherent to other cell types or debris.
- Reduction in time spent identifying sperm on slides. Happier analysts.
- Increase the number of positive samples in sexual assault cases. Corroborate victim's testimony. Increase convictions

7. PRODUCT SPECIFICATIONS FOR THE SPERMPAINT REAGENT

SpermPaint

A mixture of two fluorescent labeled monoclonal antibodies that bind to the sperm head and the sperm tail.

PRODUCT INFORMATION

Description: AlexaFluor 488 conjugated murine IgG1 monoclonal antibodies from hybridoma serum-free culture medium. Monoclonal antibody 3C6-AF is directed to the human sperm

acrosomal antigen ESP**1.** Monoclonal antibody 3A4-AF is directed to the human sperm flagellar antigen CABYR**2**.

Code Number: 3C6-AF/3A4-AF **Lot:** 093003-111003

Physical State: Liquid **Volume:** 200µl **Buffer:** Phosphate buffered saline with 50% glycerol

Antibody Concentration: 0.9 mg/ml (based on absorbance at 280nm and 494nm)

Suggested Dilution Range: 1:50-1:100 for immunohistochemistry on sperm eluted from sexual assault swabs.

Storage: The conjugate is stable at 4°C for several months. For long term storage, divide into aliquots and freeze at -20°C. AVOID REPEATED FREEZE - THAWING. PROTECT FROM LIGHT.

Appearance: Light green, clear **Clean Up:** Wash with soap and water

Health Hazards: Not listed as a toxin or carcinogen by OSHA, NTP or IARC

Potential Hazards: Free, unconjugated AlexaFluor 488 is negligible in this preparation. The complete properties of AlexaFluor 488 have not been investigated. Similar compounds are known to be chemically reactive with proteins and other biochemicals and should be treated as potentially hazardous.

.

Country of Origin: USA

Michael J. Wolkowicz, Jagathpala Shetty, Anne Westbrook, Ken Klotz, Frederike Jayes, Arabinda Mandal, Charles J. Flickinger, and John C. Herr. Equatorial Segment Protein Defines a Discrete Acrosomal Subcompartment Persisting Throughout Acrosomal Biogenesis. Biology of Reproduction 2003; 69: 735-745.

Soren Naaby-Hansen, Arabinda Mandal, Michael J. Wolkowicz, Buer Sen, V. Anne Westbrook, Jagathpala Shetty, Scott A. Coonrod, Kenneth L. Klotz, Young-Hwan Kim, Leigh Ann Bush, Charles J Flickinger, and John C. Herr. CABYR, a Novel Calcium-Binding Tyrosine Phosphorylation-Regulated Fibrous Sheath Protein. Developmental Biology 2002; 242: 236-254.

Use: FOR FORENSIC RESEARCH PURPOSES ONLY NOT FOR IN VIVO, INTERNAL OR CLINICAL USE

8. CONCLUSIONS AND RECOMMENDATIONS

SpermPaint deployment is going slow. Most labs do not have fluorescent microscope capability. Although practitioners want to incorporate the new methods they have an already overworked schedule and limited budgets. Although Congress has authorized more than $1 billion over the

next five years as part of the President's DNA Backlog Reduction Act, in talking with forensic practitioners, I sense that most of the previously allocated funds have already been targeted for new DNA sequencers, analysis of the backlog samples, personnel and for specific areas of genomics and DNA probe research. The capacity building need in fluorescence microscopy that has been occasioned by the discovery of the SpermPaint probes may require a targeted pool of funds, i.e. capital equipment funding is needed to develop this capability. Maybe something like a "Fluorescent Microscope Deployment/Utilization Act" or "Forensic DNA Fluorescence Microscopy Capacity Enhancement Act". This would benefit not only SpermPaint but other fluorescent microscopy based technologies as well.The idea for such an act needs one or more vocal champions [besides the UVA inventors].

9. AN APPROACH TO A FORENSIC DNA FLUORESCENCE MICROSCOPY CAPACITY ENHANCEMENT ACT.

Legislative Background. In 2004 Congress Passed the President's DNA Initiative, "Advancing Justice Through DNA Technology" [www.DNA.gov] authorizing more than 1 billion in funds to support capacity building in DNA fingerprinting over five years. Annual congressional appropriations support this authorization as well as providing for unique needs within the DNA fingerprinting field. For example in 2005, the federal "DNA Fingerprint Act of 2005" was introduced to allow states to upload DNA samples covered under statutory authority (such as arrestees). The National Institute of Justice serves as a key awarding agency under these acts and is proposed as recipient of the appropriation outlined below.

SpermPaint Development at Virginia. At UVA research in the human genome under a grant from the National Institute of Justice has led to the filing of recent patents on research advances in the field of forensic analysis of sexual assault evidence. The advance is called SpermPaint. SpermPaint is a fluorescent reagent comprised of monoclonal antibody probes that permit rapid identification of sperm in sexual assault evidence. The SpermPaint probes are directed to unique molecules located in the sperm head and in the sperm tail.

Impact of the Technology. SpermPaint is anticipated to impact all crime labs handling sexual assault evidence by offering improvements to current methods of staining sperm for microscopic identification as well as recovery of DNA. These advances include: 1) Rapid discernment of fluorescing sperm heads and tails against a dark background; 2) Conclusive identification of sperm heads and tails even when they are detached from one another; 3) Conclusive identification of sperm heads and tails when they are masked by adherence to other cell types and debris; 4) Extension of the post-coital interval (post-mortem interval in the case of homicide/rape) in which positive identification of sperm can be made; 5) An increased capacity to identify intact sperm flagella and sperm heads in evidence from longer post-coital intervals; 6) Methods to obtain nuclear and mitochondrial DNA fingerprints from detached sperm heads and flagella particularly at longer post-coital intervals. These performance characteristics of SpermPaints are anticipated to allow more rapid identification of sperm in microscopic fields, increase the number of conclusive identifications and thus increase the number of convictions.

Barriers to Deployment. SpermPaint requires the use of a fluorescent microscope and camera. Currently only a few forensic labs in the country are able to utilize the SpermPaint technology mainly because they do not have fluorescence microscopy capability.

Proposed Legislation. An amendment to the next appropriation bill for the Presidents DNA Initiative is proposed. The amendment would increase the NIJ allocation by $10.3 M. Budget for this initiative: Purchase of new fluorescent microscopes and cameras [351* labs X $30,000 = $10,530,000], to upgrade existing microscopes with fluorescent capability 50 X $20,000 = $1,000,000, and to develop manpower capability in the new technique [personnel for utilization/training/standardization of SpermPaint protocols: 351 X $55,000 = $19,305,000]**. Budget over 3 years = $30,835,000 or $10,278,000 per year**.*Bureau of Justice Statistics recent survey of public funded forensic labs.

10. PUBLICATIONS.

Young-Hwan Kim, Kula N. Jha, Arabinda Mandal, Geeta Vanage, Erin Farris, Phillip L. Snow, Ken Klotz, Charles J. Flickinger and John C. Herr 2005 Translation and Assembly of CABYR Coding Region B in Fibrous Sheath and Restriction of Calcium Binding to Coding Region A. Dev. Biol. 286: 46-56.

Jagathpala Shetty, Michael J. Wolkowicz, Kenneth L. Klotz, Friederike L. Jayes, Alan Diekman, Laura Bolling, V. Anne Westbrook, Erin M. Dickinson, Scott Coonrod, Charles J. Flickinger, and John C. Herr. 2003 SAMP14, a Novel, Acrosomal Membrane Associated, GPI anchored member of the Ly-6/UPAR Receptor Superfamily with a Role in Sperm-Egg Interaction. J. Biol. of Chem. Vol. 278: 30506-30515.

Soren Naaby-Hansen, Arabinda Mandal, Michael J. Wolkowicz, Sen Buer, Jacque Retief, V. Anne Westbrook, Jagathpala Shetty, Michael Cox, Friederike Jayes, Scott A. Coonrod, Kenneth L. Klotz, Leigh Ann Bush, Charles J. Flickinger, and John C. Herr. 2002 CABYR, a novel calcium binding tyrosine phosphorylation regulated fibrous sheath protein involved in capacitation. Dev. Biol. 242: 236-254.

Buer Sen, Arabinda Mandal, Michael J. Wolkowicz, Prabhakara Reddi, Jagathpala Shetty, Leigh Ann Bush, Charles J. Flickinger and John C. Herr. 2003 Cloning and Characterization of the Murine Calcium Binding, Tyrosine Phosphorylated Fibrous Sheath Protein, CABYR. Gene 310:67-78.

Arabinda Mandal, Kenneth L. Klotz, Jagathpala Shetty, Friederike L. Jayes, Michael J. Wolkowicz, Laura C. Bolling, Scott A. Coonrod, Michael B. Black, Alan B. Diekman, Timothy A.J. Haystead, Charles J. Flickinger and John C. Herr. 2003 SLLP1, A Unique, Intra-Acrosomal, Non-Bacteriolytic, c Lysozyme-Like Protein in Human Spermatozoa. Biol. of Reprod. 68:1525-1537.

Wolkowicz, M.J., Shetty, J., Westbrook, A., Bolling, L., Klotz, K., Coonrod, S., Jayes, F., Mandal, A., Flickinger, C.J., and Herr, J.C. 2003 Equatorial Segment Protein (ESP): An Alloantigenic Glycoprotein that Mediates Sperm-Egg Interactions and Provides Evidence for an

Equatorial Segment Subcompartment in Acrosomal Vesicle Stage Spermatids. Biol. Reprod. 69: 735-745.

Wang Z, Zhang Y, Mandal A, Zhang J, Giles FJ, JC Herr, Lim SH. The spermatozoa protein, SLLP1, is a novel cancer-testis antigen in hematologic malignancies. 2004 Clinical Cancer Research 10: 6544-50.

Susan Sleight, Patricia Miranda, Nia-Washington Plaskett, Bernhard Maier, Jeff Lysiak, Heide Scrable, John C. Herr and Pablo E. Visconti. 2005 Isolation and proteomic analysis of mouse sperm detergent-resistant membrane fractions: evidence for dissociation of lipid rafts during capacitation. Biology of Reproduction 73(4): 721-9.

V. Anne Westbrook, Pamela D. Schoppee, Geeta R. Vanage, Ken L. Klotz, Alan B. Diekman, Charles J. Flickinger, Michael A. Coppola, John C. Herr 2006 Spermatid Nuclear Envelope Morphogenesis Evidenced by Localization of the SPAN-X Cancer/Testis Antigen During Human and Chimpanzee Spermiogenesis. Molecular Human Reproduction 12 (11): 703-716.

Nirmala Jagadish, Ritu Rana, Ramasamy Selvi, Deepshikha Mishra, Manog Garg, Shikha Yadav, John C. Herr, Katsuzumi Okumura, Akiko Hasegawa, Koji Koyama, Anil Suri. 2005 Characterization of a novel human sperm associated antigen 9 (SPAG9) having structural homology with α-Jun NH2-terminal kinase interacting protein. Biochemical Journal 389(Pt 1):73-82.

Clara I. Marin-Briggiler, Kula Nand Jha, Olga Chertihin, Mariano G. Buffone, John C. Herr, Moniza H. Vazquez-Levin and Pablo Visconti. 2005 Evidence of the presence of calcium/calmodulin-dependent protein kinase IV in human sperm and its involvement in motility regulation. J. Cell Science 118 (9): 2013-2022.

Young-Hwan Kim, Kula N. Jha, Arabinda Mandal, Geeta Vanage, Erin Farris, Phillip L. Snow, Ken Klotz, Charles J. Flickinger and John C. Herr 2005 Translation and assembly of CABYR coding Region B in fibrous sheath and restriction of calcium binding to coding Region A. Dev. Biol. 286: 46-56.

Herrero MB, Mandal A, Digilio LC, Coonrod SA, Maier B, JC Herr. Mouse SLLP1, a sperm lysozyme-like protein involved in sperm-egg binding and fertilization. 2005 Developmental Biol. 284: 126-142.

Scott Coonrod, Alejandra Vitale, Chongwen Duan, John Herr, Sarah Bristol-Gould and Erwin Goldberg. 2006 Expression of Testis-Specific Lacatate Dehydrogenase (LDH-C_4; Ldh3) in Murine Oocytes and Preimplantation Embryos. J. Androl. 27 (4): 502-509.

B. Xu, Z. Hao, K. N. Jha, L. Digilio, C. Urekar, YH. Kim, W. He, S. Pulido, C. J. Flickinger, J. C. Herr 2006 Validation of a testis specific serine/threonine kinase [TSSK] family, and the substrate of TSSK1 & 2, TSKS, as contraceptive targets. Reproduction [in press].

Bingfang Xu, Michael Coppolla, John C. Herr, and Michael P. Timko 2006 Expression of a recombinant human sperm-agglutinating miniantibody in tobacco (*Nicotiana tabacum* L.). Reproduction [in press].

Young-Hwan Kim, Gerhard Haidl, Ursula Egner and John C. Herr Compartmentalization of Glycolytic Enzymes and a Unique ADP/ATP Carrier Protein AAC4 (Sperm Flagellar Energy Carrier, SFEC) in the Fibrous Sheath of the Sperm Flagellar Principal Piece. 2007 Dev Biol. 302: 463-476.

11. REFERENCES.

1. K A Collins, A T. Bennett. 2001American Journal of Forensic Medicine and Pathology 22(3):228-232.

2. I.J. Pandya, Jack Cohen. 1985. Fertility and Sterility 43: 417-421.

3. L.A. Thompson, C.L.R. Barratt, A.E. Bolton, and I.D. Cooke 1992 American Journal of Reproductive Immunology 28:85-89.

4. Budowle B, Allard MW, Wilson MR, and Chakraborty R (2003) Annu Rev Genomics Hum Genet 4:119-141.

5. Díez-Sanchez C, Ruiz-Pesini E, Lapena AC et al. (2003) Biol Reprod 68:180-185.

6. Anslinger K et al. (in press) MtDNA investigations after differential lysis. J For Sci.

7. Danielson PB, Kristinsson R, Shelton RJ, and LaBerge GS (2005) Expert Rev Mol Diagn 5:1-11.

8. Gabriel MN, Huffine EF, Ryan JH et al (2001) J For Sci 46:247-253.

9. S Naaby-Hansen, et al 2002, Dev. Biol. 242: 236-254.

10. Wolkowicz, M.J., et al 2003 Biol. Reprod. 69: 735-745.

11. Buer Sen, et al. 2003. Gene 310:67-78.

12. L. Gilmer, et al 2004 Composition for Identifying Sperm for Forensic Applications. US Patent and Trademark APPLICATION (ATTORNEY DOCKET # 009520-01).

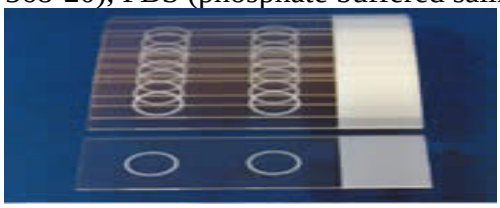
12. APPENDIX.

SPERM PAINT PROTOCOL

Methods of slide preparation other than that outlined below may prove compatible with SpermPaint reagents. Testing other protocols is encouraged, but success should be first demonstrated with the protocol presented here. It has been extensively tested and is recommended to obtain results as illustrated in figures 1, 2, and 3.

Materials Provided:
SpermPaint, four (4) 100µl aliquots. (Each aliquot will stain 25-50 slides.)
Normal goat serum, four (4) 500µl aliquots. (Blocking agent)
Antifade mounting media: One dropper bottle, 2 ml.

Materials not Provided:
Fluorescent microscope with a 490-519nm excitation filter (also used for fluorescein, FITC), slides, fluorescent antibody microscope 2 ring slides (BD micro slides-Fisher cat# 12-568-20), PBS (phosphate buffered saline), coverslips (Fisher cat#12-548-5E) and nail polish.



Method:

1. Rehydrate sample collected on cotton-tipped swab in 0.5ml PBS per swab for 20 minutes, agitate manually at 5 minute intervals to release cells into PBS. Apply 25 microliters of PBS suspension to microscope slide and air dry at room temperature overnight or on a slide warmer for 2-3 hours, not to exceed 37°C in order to preserve antigenic sites on the sperm.

2. Sample on slide is rehydrated by overlaying 50-100µl PBS directly onto sample area and incubating for five minutes.

3. Aspirate PBS with pipet or vacuum source and apply SpermPaint (diluted 1:25 in PBS with 10% normal goat serum) to sample on slide. Incubate in a closed container with a wet paper towel on the bottom of container to prevent the slide from drying out. Best results are obtained with incubation overnight at 4°C. Good results are also obtained incubating for two hours at room temperature.

4. Aspirate to remove SpermPaint , wash five times with PBS. Do not use coplin jar to wash slides as it interferes with the antifade mounting medium.

5. Apply anti-fade reagent to the sample and cover with a coverslip and seal with nail polish. Store slides flat in a covered folder at 4°C.