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Development of an Immuno-Magnetic Procedure for the Separation of Spermatozoa from Vaginal Epithelial Cells

FINAL REPORT

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Abstract

Forensic biology laboratories have long been searching for a more efficient, automatable method for processing sexual assault evidence. With some minor modifications a manual differential lysis extraction first described over 20 years ago is still the most commonly used method for separating sperm cells for vaginal epithelial cells. In this project, the goal was to utilize immuno-magnetic bead "capture and release" chemistry to specifically bind spermatozoa and sperm heads while all other cell types are removed. Such an assay could easily be moved to a robotic platform and allow for a more efficient workflow.

Several promising sperm membrane specific antigens were identified based on published research and the human gene compendium. Target proteins for the antibodies tested included ADAM2, AKAP3, MFGE8, MOSPD3, Ropn1l, SPAM1, and UBAP2L. One type of capture chemistry tested in this study was magnetic beads covered with Protein G, which exhibits a high affinity for immunoglobulin (IgG) and will bind this antibody domain. The other bead type was covered with streptavidin thus reacting with any biotinylated antibodies. Two different biotin conjugates (DSB-X Biotin and EZ-link sulfo-NHS-LC-Biotin) were tested for this labeling step.

Varying numbers of single source sperm cells and female vaginal epithelial cells were processed with different antibodies and both types of beads. In order to separately evaluate the captured cells bound to the beads and the remaining cells still in solution, both the "capture" fraction and the "supernatant" fraction were subjected to DNA extraction and quantitation with a real time PCR assay specific for a human DNA target and a male Y-chromosome sequence. Overall this project failed to demonstrate sperm cell specific enrichment for either magnetic bead capture assay. Protein G beads were shown to retain DNA, but in a non-specific fashion including vaginal epithelial cells and control samples with no antibody addition. For the streptavidin coated beads almost the entire recovered DNA resided in the supernatant fraction that was taken off the immobilized beads. Based on the results for single source samples the project never matured to the stage, where real cell separation for sperm/vaginal cell mixtures was attempted.

The DNA recovered by the Protein G beads is probably due to unplanned lysis of cells in suspension and unspecific retention of DNA and DNA-protein complexes by the bead surface. Microscopic examination failed to detect any intact cells adhering to the Protein G beads. Both biotinylation strategies were unsuccessful in capturing sperm cells on the streptavidin coated beads. Again, except for a single isolated observation, microscopic examination failed to detect spermatozoa-bead connections. Antibody quality and lack of antigen specificity could have played a role, but at least two of the antibodies had shown a positive sperm cell agglutination reaction. Streptavidin covered beads are routinely used for cell sorting and this assay had been successfully used for sperm separation with the MOSPD3 antibody. The failure to duplicate

these results is most likely due to technical problems, especially with the biotinylation step and insufficient antibody labeling.

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Executive Summary

Introduction

Forensic biology laboratories have long been searching for a more efficient, automatable method for processing sexual assault evidence. The most commonly used method is still the manual differential lysis extraction first described over 20 years ago (1). In this project the goal was to utilize magnetic bead capture and release chemistry to specifically bind spermatozoa and sperm heads while all other cell types are removed. This approach is routinely used in many clinical and basic research assays aiming at specific cell types in whole blood or cell culture specimen (for example 2-4). Several commercially available products offer universally applicable beads that enable research teams to use their own antibodies. New England Biolabs has a magnetic bead that is covalently coupled to a truncated form of recombinant Protein G, which exhibits a high affinity for immunoglobulin (IgG) from many species including human, rabbit, mouse and sheep (5, 6). Other types of customizable beads are being offered by Invitrogen Lifetechnologies (7). These beads are either coated with streptavidin, thus reacting with any biotinylated antibodies, coupled with secondary antibodies against immunoglobulin domains, or epoxy coated. The epoxy coated surface immobilizes ligands containing amino, thiol and hydroxyl functional groups; a property that can be used to cover the beads with any custom made antibody (8).

Early work on the forensic application of this type of assay includes the use of epoxy beads and photoactivation by Eisenberg (9, 10), and IgG antibody coupled beads by Anslinger et al (11). While there were some encouraging results, neither project resulted in either a peer reviewed publication or an optimized forensic laboratory assay for sperm capture. The latest published sperm-magnetic bead results were generated using the biotin-streptavidin bead approach and an antibody against MOSPD3 (motile sperm domain containing protein 3) (12). This antibody is thought to be involved in spermatogenesis (13) and encodes a multi-pass membrane protein with a major sperm protein (MSP) domain (14). This assay was shown to effectively separate fresh sperm cells from female buccal cells but displayed decreased recovery for sperm cells on vaginal swabs that had been stored for up to 10 days (12).

Research Design

Experiments in the present project tested a panel of five different sperm membrane specific antibodies identified via the human gene compendium and a review of the relevant literature for their ability to capture human sperm via the New England Biolabs Protein G assay. This bead type was chosen based on promising preliminary results for a similar bead type also from New England Biolabs (15). In a later stage of the project, a two more antibodies were added to the panel and a second type of bead, a streptavidin coated Lifetechnology bead, was utilized. Planned experiments included: antibody reactivity testing, sperm capture from single source

samples, sperm capture from mixtures, optimization of the magnetic bead assay and comparison to current manual differential lysis methods. In order to separately evaluate the captured cells bound to the beads and the remaining cells still in solution, both the "capture" fraction and the "supernatant" fraction were subjected to DNA extraction and quantitation with a real time PCR assay specific for a human DNA target and a male Y-chromosome sequence. To prove the suitability of the recovered DNA for subsequent STR analysis, DNA fractions for several experiments were typed using the AmpFlSTR Identifiler Plus Kit from Applied Biosystems – Lifetechnologies.

The following antibodies were included in the study: Ferrtilin β ADAM 2) a single-pass type I sperm membrane disintegrin and metalloproteinase domain-containing protein involved in sperm-oocyte binding (14, 16, 17); sperm adhesion molecule (SPAM1), a GPI-anchor hyaluronidase localized on the sperm surface and inner acrosomal membrane involved in sperm-zona pellucida adhesion (14, 16); MFGE8 or SED1 (secreted protein containing EGF repeats and Discoidin/F5/8 complement domains) which coats sperm as they traverse though the epididymis and is required for sperm binding to the egg coat (14, 18); ubiquitin-associated protein 2 like protein (UBAP2L) interacting with the ZP3 receptor (14, 19), and AKAP3 or A kinase anchor protein 3, which is expressed in spermatozoa and localized to both acrosomal region of the sperm head as well as the principal region of the flagella (14, 20). MFGE8 and ADAM2 were tested both in monoclonal and polyclonal form, resulting in a panel of seven antibodies total. The additional antibodies later added to this panel were specific to AKAP associated Sperm Protein or Ropn1l (21, 22) acting on sperm motility and MOSPD3 (motile sperm domain containing protein 3), which is thought to be involved in spermatogenesis (12, 13) and encodes a multi-pass membrane protein with a major sperm protein (MSP) domain (14).

The project can be divided in four phases: phase 1 – single source experiments with the full initial panel of seven antibodies and Protein G beads; phase 2 - single source experiments with a reduced panel of the four most promising antibodies and Protein G beads; phase 3 - duplication of the phase 2 single source experiments with fresh biological specimen and two of the antibodies, and phase 4 – single source experiments using streptavidin coated beads and three of the original panel antibodies, a new candidate Ropn1l, and the antibody to MOSPD3 that was selected based on the 2014 publication on sperm capture (12).

Results

Protein G bead results were originally interpreted as displaying specificity differences between the seven antibodies. The tested samples came from two different semen donors with normalized cell counts from 20 to 20,000 sperm. Four of the seven antibodies samples had more DNA in the sperm cell capture fraction than in the supernatant. These four antibodies were further investigated for both semen donors and different amounts of antibody, including a "no antibody"

control where 1xPBS was substituted for the antibody addition. Several of the samples, including the “no antibody” controls displayed a higher DNA yield in the capture than the supernatant fraction. This unspecific binding and DNA recovery was confirmed through two experiments with female vaginal epithelial cells. Most of the DNA was found associated with the Protein G beads and present in the capture fraction. Phase 3 duplicated the phase 2 results for MFGE8 and UBAP2L and fresh biological samples. Again, most if not all of the recovered DNA was in the bead capture fraction for both the male and female cell types and including the “no antibody” control.

The streptavidin-coated beads successfully utilized in the publication by Li et al (12) are available as the Dynabead FlowComp Flexi kit from Invitrogen-Life Technologies Carlsbad, CA, USA). The biotin labeling chemistry included in this kit is based on DSB-X biotin, which was used to biotinylate the antibody to MOSPD3 introduced by Li et al (12) and three of the antibodies in the original panel. Single source experiments using the Dynabead FlowComp Flexi beads were performed for two fresh semen samples and two new female vaginal swab donors. For this type of beads for all of the antibodies and the “no antibody” controls almost none of the DNA resided in the bead capture fraction. The amount of DNA recovered in the supernatant correlated to the positive control samples, but there was no enrichment through bead capture. This lack of capture result was duplicated for MOSPD3 and Ropn1l using an alternative biotinylation assay, the EZ-Link Sulfo-NHS-LC-Biotin kit from Thermoscientific utilized by Li et al (12).

Overall this project failed to demonstrate sperm cell specific enrichment for either magnetic bead capture assay. Protein G beads were shown to retain DNA, but in a non-specific fashion including vaginal epithelial cells and control samples with no antibody addition. For the Dynabead FlowComp Flexi kit almost the entire recovered DNA resided in the supernatant fraction that was taken off the immobilized beads.

Discussion

For the New England Biolabs Protein G beads the problem was not a lack of DNA recovery, but rather the unspecific retention of DNA from sperm and non-sperm cells, with and without the addition of sperm membrane receptor specific antibodies. It is unlikely that the beads were binding whole cells. No cell to bead adhesion was seen during microscopic examination of a Protein G sperm extraction bead fraction after bead immobilization and washing. While there has been some discussion on cell free DNA (23), it is more likely that cells were lysed during the magnetic bead incubation step. Cells should have been intact after suspension in phosphate buffered saline, but the Protein G sodium phosphate binding buffer combined with the mechanical mixing in the presence of metal beads may have disrupted not only epithelial cell but also sperm head membranes. These magnetic beads may have an affinity to DNA or DNA-

histone complexes either via the Protein G coating or possibly gaps in this coat. No experiments with cell lysates or previously extracted DNA were performed to explore this. Anslinger at el working with anti-IgG antibodies also reported on this unspecific extraction of female cell DNA (11).

For the assay with streptavidin coated beads, one can think of three possible areas where this assay could fail: The first factor that would prevent any specific binding is the choice and the quality of the purchased antibodies. Most antibody manufacturers only test their antibody performance for Western Blot applications and researchers have encountered vast differences in quality and binding capability for the same type of antibody from different companies. The agglutination tests showed a strong reaction at least to two antibodies in the panel (AKAP3 and MOSPD3), but this did not translate into successful magnetic bead capture.

Under the assumption that antibodies were sufficiently labeled, sufficiently specific to an accessible sperm membrane antigen, and buffer and temperature conditions were suitable for efficient binding, the last factor prohibiting cell enrichment is the strength of the non-covalent biotin-streptavidin bond and its reaction to shearing effects during the required wash steps. As described by Eisenberg (10) agitation and stringent washing led to loss of previously bound material for the epoxy-antibody bead conjugates, which was the reason for adding the stabilizing photolysis step. It remains a fact that many other research teams have successfully recovered their target cell population (2-4). Overall performance will also be affected by the density of antigens available on the target for binding, number of biotin molecules incorporated per antibody and density of streptavidin receptors on the beads. For many of these applications there is abundant starting material and a certain loss of cellular material is expected and tolerated, but is unlikely that lack of thermodynamic stability and physical disruption of already formed bonds is the explanation for the total failure to capture sperm cell DNA on the streptavidin coated beads.

The third source of failure is biotin labeling reaction which could have been unsuccessful leading to unlabeled and thus ineffective antibodies. The initial labeling chemistry used in this study was DSB-X biotin succinimidyl ester, which was part of the Dynabead FlowComp Flexi kit and was run for 1.5 hours as instructed by the manufacturer. Li et al (12) deviated in this step and labeled the MOSPD3 antibody with sulfo-NHS-LC-biotin obtained from Pierce Protein Biology Products, Thermo Scientific, Rockford, IL, USA; the labeling reaction was allowed to incubate for 72 hours. While there were no obvious issues with the DSB-X biotin succinimidyl ester reaction, it must be stated that the actual binding of the biotin label was not confirmed via Western blot and labeled streptavidin probes. To address this possible source of failure, MOSPD3 and Ropn1l were labeled following Li et al (12) as closely as possible and the labeling efficiency was monitored using a biotin quantitation kit. The quantitation revealed low incorporation and the subsequent capturing experiments failed to show any enrichment.

Overall the failure to duplicate the results presented by Li et al (12) may be based on a combination of factors with antibody quality and some of the buffers and incubation conditions failing to facilitate optimal binding. But at least for the EZ-Link sulfo-NHS-LC-biotin kit reaction, the most likely step to have failed in our hands was the antibody biotinylation, which means the work presented here was compromised by technical issues and cannot be used to decide on the overall scientific merit of the magnetic bead sperm capture method. Unfortunately it was not possible to spend more time on optimizing this critical step.

Conclusions and implications for further research

This study clearly demonstrated that Protein G beads are not suitable for magnetic bead based sperm separation. For the streptavidin coated beads, the failure to duplicate the results described by Li et al (12) may be based on a combination of factors with antibody quality and some of the buffers and incubation conditions being possible factors. But at least for the EZ-Link sulfo-NHS-LC-biotin kit reaction the most likely step to have failed in our hands was the antibody biotinylation, which means the work presented here was compromised by technical issues and cannot be used to decide on the overall scientific merit of the magnetic bead sperm capture method.

The paper by Li et al is the only peer reviewed publication on the successful recovery of sperm cells and male DNA from mixtures with female cells using streptavidin covered beads (12). But they also found that the assay was better suited for fresh sperm and describe their success rate declining rapidly for dried vaginal swabs after short storage periods. It is unclear, if further research would be able to improve on these results. Surface antigens are easily compromised and capture release assays are always accompanied by a certain amount of loss. Forensic evidence by nature does not consist of pristine and abundant cells and even with very effective antibodies and under optimized conditions magnetic bead assays will not be able to recover the highest possible amount of male DNA. This makes the idea of an automated immuno-magnetic sperm capture assay problematic for compromised samples and evidence with low sperm content. If a working assay could be developed its application is likely to be limited to fairly fresh and abundant samples.

Introduction

Statement of the problem

In the United States, sexual assault occurs every two minutes and more than 200,000 women are sexually assaulted each year (24, 25). The case backlog of unanalyzed sexual assault cases has been deemed a human rights issue and there have been demands to make the timely processing of this evidence a priority for criminal justice policy for many years (26). The most probative evidence in such cases is the identification of the semen donor from spermatozoa recovered from vaginal swabs. The mixed nature of the sample (semen from the suspect and vaginal epithelial cells from the victim) complicates DNA analysis. In these mixed samples, vaginal epithelial cells vastly outnumber the sperm and separation of the male and female components in these samples is critical for subsequent genetic analysis by PCR-STR. Overall there has been a lot of progress in forensic DNA evidence processing and almost all techniques have been getting faster, more sensitive and more discriminatory (27) with one notable exception. The DNA extraction from mixed samples from sexual assault evidence using the differential extraction procedure (1) has remained virtually unchanged for the past 20 years. This separation procedure is time-consuming and labor intensive; the separation of the male and female fractions is oftentimes incomplete which complicates the interpretation of the DNA typing results. A simple, robust, cost and time efficient method for sperm cell separation from sexual assault swabs would be of practical forensic value.

The differential extraction procedure (1) is a physical-chemical method that exploits the differences in membrane stability between the sperm and vaginal epithelial cells to separate the cellular components. This multi-step process involves lysis of epithelial cell membranes using mild conditions and collection of sperm cells (predominantly heads) by centrifugation. The pelleted sperm cells are washed several times using phosphate buffered saline (PBS), resuspended and lysed in a buffer containing dithiothreitol (DTT). The DNA in the resultant sperm and epithelial cell fractions is extracted using a method based on either an organic resin (Chelex, BioRad, Hercules, CA), silica (QIAamp, Qiagen, Valencia, CA), organic phase separation (phenol:chloroform) or paramagnetic beads (DNA IQ, Promega, Madison, WI). The separation procedure is time-consuming (approximately two hours and labor intensive. Separation of the male and female fractions is oftentimes incomplete and thus complicates the interpretation of the DNA typing results. The repeated washes can result in sperm cell loss and the multiple steps increase the risk of contamination. Several alternative methods of cell separation have been proposed.

Literature review

Physical, Chemical and Enzymatic Separation Techniques

Schoell et al (28) used a fluorescence-activated cell sorting method to separate sperm and vaginal cells. The method has limited applicability to forensic casework since it would require a change in the method of collection of evidentiary samples from vaginal swabs to vaginal lavages. Laser

capture micro-dissection (LCM) has also been used to separate sperm cells from vaginal epithelial cells (29-31). Although LCM provides a very precise, clean separation, the technique is labor-intensive, time-consuming and costly and thus not amenable to high- throughput analysis of casework samples. Filtration-based methods of separation have also been developed (32, 33). The separation was incomplete and DNA from spontaneously lysed vaginal cells was present in the sperm fraction. Clogging of the membrane by debris (cotton fibers from the vaginal swab) was also problematic. Horsman et al (34) used a micro-fabricated device to separate sperm and vaginal epithelial cells. Although the method shows promise, the collection (30 min/sample) is time-consuming. Bienvenue, et al (35) took the microfluidic device a step further by developing a microchip- based cell lysis and extraction model. Full DNA profiles were obtained. While this appears to be a successful approach, both extraction and separation techniques are labor intensive.

Selective degradation of female DNA in mixed samples via nuclease (DNase I) digestion shows promise (36). However, the method is limited due to the long digestion incubation time (5 hrs.) prior to DNA purification and thus not amenable to high-throughput analysis of casework samples. The Differex™ System developed by Promega (Madison, WI) uses phase separation and differential centrifugation to separate sperm and epithelial cells. Although the method produces cleaner separations than the conventional differential extraction method, the technique is still labor intensive (37, 38), as is another optimized differential lysis protocol utilizing a modified elution buffer (39). Casework samples need a more rapid and possibly automated technique. Haak, Porsche, Vollack, Zimmermann, & Pflug (40) developed a semi-automatic, magnetic-bead based DNA extraction method using the ChargeSwitch® Forensic DNA Purification Kit (Invitrogen, Carlsbad, CA) and two Te-MagS magnetic bead separation modules (Tecan, Durham, NC). The system was interfaced with a Freedom EVO 150 liquid handling workstation (Tecan, Durham, NC) equipped with an eight-channel liquid handling arm and a PosID barcode sample identification device (Tecan, Durham, NC). This completely automated system was compared to the manually based GEN-IAL First Magnetic Forensic kit (GEN-IAL GmbH, Troisdorf, Germany) method. While both methods produced similar DNA yields, neither produced a definitive separation of cell types, leaving an “enormous excess” of female DNA (38). This is a significant disadvantage when working with casework samples.

More recently the DNA team working for the California Department of Justice published a high-throughput semen evidence workflow that seems promising (41). The method relies on the observed propensity of sperm cells to adhere to a substrate such as a vaginal swab, which makes it possible to lyse cells and remove non-sperm DNA using an initial alkaline lysis step. Subsequent DNAase digestion is used to eliminate remaining non-sperm DNA on the soaked substrate prior to the sperm lysis step. The authors describe increased yields over the standard DTT based differential lysis but caution about possible loss of sperm DNA for aged swabs and other stain substrates (41).

Sperm Surface Receptors

An alternative to the methods discussed above is a separation procedure based on the antigenic properties of human spermatozoa. Anti-sperm antibodies have been studied extensively in the field of human development and reproduction. Sperm cells possess a high degree of cell surface differentiation. Each region is designated for a specific function that is mediated by antigens located on the head, acrosome, equatorial, midpiece, and tail sections of spermatozoa (42).The complex process of sperm binding to the zona pellucida of oocytes has been a highly researched topic in the field of reproduction and development. Human zona pellucida (ZP) glycoproteins have been found to play the most important role in sperm binding. The human zona pellucida is a protein matrix surrounding the oocyte which contains four glycoproteins – ZP1, ZP2, ZP3 and ZP4, respectively. ZP3 has been identified as the primary sperm binding protein and plays a complex role in the initial binding of the sperm head ZP3 receptor (ZP3R) to the zona pellucida and subsequent induction of the acrosomal reaction necessary for fertilization (43).

While the ZP3 receptor has been shown to play a critical role in sperm-zona pellucida binding, it is only one component of a complex reaction that mediates sperm fusion to the oocyte. Ferrtilin β (ADAM 2) is a single-pass type I sperm membrane disintegrin and metalloproteinase domain-containing protein involved in sperm-oocyte binding (14, 16, 17). Sperm adhesion molecule (SPAM1/PH-20) is a GPI-anchor hyaluronidase localized on the sperm surface and inner acrosomal membrane involved in sperm-zona pellucida adhesion (14, 16). MFGE8 or SED1 (secreted protein containing EGF repeats and Discoidin/F5/8 complement domains) is an EGF repeat and discoidin domain protein that coats sperm as they traverse though the epididymis and is required for sperm binding to the egg coat (14, 18). Ubiquitin-associated protein 2 like (UBAP2L) protein interacts with the ZP3 receptor (14, 19). AKAP3 is also known as a kinase anchor protein 3, is expressed in spermatozoa, and localized to both acrosomal region of the sperm head as well as the principal region of the flagella (14, 20). A similar target is the human AKAP associated sperm protein Ropn1l (21, 22). Motile sperm domain containing protein 3 (MOSPD3) is thought to be involved in spermatogenesis (12, 13) and encodes a multi-pass membrane protein with a major sperm protein (MSP) domain (14).

Theoretically the antigenic properties of spermatozoa can be used to develop methods applicable to the field of forensic science. It should be possible to use antibodies directed against sperm surface antigens to capture sperm cells and facilitate the separation of cellular material in mixed samples.

Immunological Separation Techniques

Several authors have explored magnetic bead separation procedure based on the antigenic properties of human spermatozoa as an alternative to the physical, chemical and enzymatic methods discussed above (9, 10, 12, 44-47). Eisenberg (9, 10) developed an antibody-based separation method using epoxy coated magnetic beads with covalently bound sperm-specific antibodies to capture spermatozoa. Although promising for casework, the technique has several

limitations. First, the three monoclonal anti-sperm antibodies used were directed against antigens located on the tail, acrosome and acrosomal cap. In most instances only sperm heads are isolated from vaginal swabs- the tails are frequently degraded. Acrosomal antigens are displayed on the surface of capacitated and acrosome-reacted sperm cells and not in ejaculates (48). While the antibody coated beads were specific to sperm cells, the strength of the bond was not sufficient to retain the sperm cells during the required wash steps. This was addressed by photoaffinity labeling of the antibody- magnetic bead complex and photoactivation to form covalent sperm-antibody bonds. Although covalent bond formation increased the avidity of the antibodies, the effect was mitigated by a reduction in the affinity of the antibodies. Fewer sperm were captured but those captured were tightly bound (9, 10). A different choice of antibody may maximize the binding and retention of sperm.

Anslinger, Bayer, Danilove, & Metzger (11) devised an immuno-magnetic bead procedure using monoclonal antibodies (mAbs) against the testicular isoform of the angiotensin-converting enzyme (tACE). This antigen is located on the neck, mid-piece and flagella of the sperm. These antibodies were coupled to Mouse IgG Dynabead (Invitrogen - Lifetechnologies, Carlsbad, CA) magnetic bead particles. The ability of each of three mAbs to separate sperm cells from vaginal epithelial cells from dried swab samples was tested. Results demonstrated a lack of antibody binding due to loss of sperm midpiece and flagellum (11). However, positive results were obtained from vaginal swabs stored in PBS buffer. This is an unrealistic situation because casework samples are stored dry and are typically days to months old. Also, depending on the ratio of cells in the mixed samples, most male DNA profiles showed varying amounts of female alleles. Overall, a major male component was found in samples that contained at least 10^5 sperm cells. This is a large number of cells and highly unlikely to be found in casework samples. Again, there is the potential that this assay can be optimized using different antibodies.

A different type of magnetic bead coated with Protein A (New England Biolabs, Ipswich, MA) was used in unpublished thesis research at John Jay College of Criminal Justice (15). Protein A displays a high affinity for immunoglobulins (IgG) from several species including rabbit and mouse and will thus bind to antibodies produced in these animals. Spermatozoa were incubated with polyclonal rabbit anti-human sperm antibody, anti-ZP3R (Aviva Systems Biology, San Diego, CA), directed against sperm zona pellucida glycoprotein 3 receptor (43) and the antibody titer was determined to be 1:256. All samples tested were single source and the endpoint determination in these studies was the amount of DNA recovered from Chelex extracts of captured sperm and buccal cell samples. The initial results confirmed the specificity of the antibody. The Protein A beads could be shown to capture more male than female DNA but overall most of the DNA was recovered in the wash fraction and many samples yielded only partial DNA profiles (15).

The latest research describing successful sperm capture using a magnetic bead procedure was published in 2014. Li et al (12) employed biotinylated monoclonal antibodies towards motile

sperm domain protein 3 (MOSPD3) and streptavidin coupled beads available in the Dynabead FlowComp Flexi kit (Invitrogen - Lifetechnologies, Carlsbad, CA). They tested fresh sperm and female buccal epithelial cells mixed at various ratios as well as dried semen containing vaginal swabs. The team was able to microscopically demonstrate magnetic bead adhesion to both the sperm head and the tail region. They obtained full male single source STR profiles from all mixtures containing 10^5 sperm cells per mL and good quality partial profiles for 10^3 and 10^4 sperm cells per mL. The dried vaginal swabs seem to have been real casework samples and were microscopically examined for the presence of spermatozoa. The authors did not define if "successful detection" again meant single source male DNA profiles, but it is stated that successful detection was correlated to storage time. While dried swabs stored for only 1 day had 100% successful detection, this rate decreased to16.67% for swabs preserved for 10 days (12).

Rationale for the Research

Immunological cell separation techniques are widely used for basic and clinical research applications and show the same promise for forensic evidence, especially for the separation of sperm from vaginal epithelial cells. In order to allow for stringent wash steps and successful removal of epithelial cells a cell capture method must combine two features. Antibodies must display a high level of specificity towards antigens not only reliably present on the sperm head membrane, but also reactive and preserved in dried specimen. The antibody-sperm complex must form a strong bond to the chosen magnetic bead. It was anticipated that it should be possible to optimize one or more of these immuno-magnetic methods and thus provide a more efficient, rapid, and cost-effective alternative to the current differential lysis methods.

Based on published data the following antibodies were selected for further testing: antibodies directed against ZP3R, ADAM2 -fertilin β, SPAM1, MFGE8-SEDI, AKAP3, UBAP2L, and a later stage human AKAP associated sperm protein or Ropn1l and MOSPD3. Unfortunately the ZP3R antibody was no longer commercially available and had to be eliminated from the study. This project employed Protein G magnetic beads (New England Biolabs, Ipswich, MA, USA) instead of the previously tested Protein A beads to take advantage of the higher reported IgG affinity for goat based antibodies (9). For phase 4 the bead assay to be evaluated was the Dynabead FlowComp Flexi kit (Invitrogen - Lifetechnologies, Carlsbad, CA) successfully used by Li et al (12). This antibody bead bond is based on the high affinity biotin-streptavidin protein-ligand interaction (12). Planned experiments included: antibody titer determination, optimum amount of anti-sperm antibodies, sperm capture from single source samples, DNA extraction and quantitation, sperm capture from mixtures, DNA amplification and genotyping, optimization of the magnetic bead assay and comparison to manual differential lysis employing a Chelex or Differex (37, 38, 47) approach.

Material and Methods

Samples

Sperm samples, vaginal swabs and buccal reference swabs were obtained from three male and four female volunteers. Volunteer recruitment and sample collection procedures had been approved by the City University of New York Institutional Review Board. In addition to the volunteer samples, two single source fresh semen samples were purchased from Lee Biosolutions, St. Louis, MO, USA. Semen samples were divided into 100µL aliquots and stored refrigerated for up to two weeks and at -20°C for long-term storage. The two fresh semen samples were also used to produce 10µL semen stains on cotton material and cotton swabs. These stains and swabs were individually packaged in coin envelopes and stored at room temperature. Vaginal swabs were air dried and stored under refrigeration; epithelial cells were obtained from the vaginal swabs by incubation in phosphate buffered saline (PBS) at room temperature with agitation (Eppendorf Thermomixer, Daigger, Vernon Hills, IL) for 2 hrs. to dislodge the cells from the substrate. The cell suspensions and swabs were stored in separate sterile tubes at 4 ºC.

Cell counting was performed by staining aliquots of spermatozoa and vaginal epithelial cell suspensions with 1X phosphate buffered saline-Methylene Blue (PBS-MB) solution (4:1) to visualize the cells and by using a Neubauer® hemocytometer.

Antibodies

Antibodies were obtained from the following sources: Abnova, Taipei, Taiwan: SPAM1 (mouse polyclonal), MFGE8 (mouse polyclonal and monoclonal), ADAM2 (mouse polyclonal and monoclonal), UBAP2L (goat polyclonal). Antibodies-Online Inc, Atlanta, GA, USA: AKAP3 (rabbit) and Abcam Inc, Cambridge, MA, USA: MOSPD3 (rabbit polyclonal), AKAP associated Sperm Protein or Ropn1l (rabbit monoclonal and polyclonal).. Antibody storage buffers were checked for sodium azide and bovine serum albumin (BSA) concentrations. No pre-purification of antibodies was performed since all levels were found to be below the critical thresholds for the two biotinylation assays employed.

Table 1 lists sources and catalog numbers for all antibodies

Table 1 Antibody information

Antibody	Origin	Company	Catalog Number
SPAM1	mouse polyclonal	Abnova, Taipei, Taiwan	H00006677-A01
MFGE8	mouse polyclonal	Abnova, Taipei, Taiwan	H00004240-A01
MFGE8	mouse monoclonal	Abnova, Taipei, Taiwan	MAB3634
ADAM2	mouse polyclonal	Abnova, Taipei, Taiwan	H00002515-M02
ADAM2	mouse monoclonal	Abnova, Taipei, Taiwan	H00002515-A01
UBAP2L	goat polyclonal	Abnova, Taipei, Taiwan	PAB22848
AKAP3	rabbit	Antibodies-Online Inc, Atlanta, GA	ABIN652454
MOSPD3	rabbit polyclonal	Abcam Inc, Cambridge, MA	GR184886
UBAP2L	rabbit polyclonal	Abcam Inc, Cambridge, MA	GR169595
Ropn1l	rabbit polyclonal	Abcam Inc, Cambridge, MA	GR189438
Ropn1l	rabbit monoclonal	Abcam Inc, Cambridge, MA	GR130918

Antibody titer was determined using the modified slide agglutination test (50, 51) at a constant sperm concentration and Terasaki optical microtiter plates. A series of two-fold dilutions (1:1 to 1:256) of the anti-sperm antibody was made using 1X phosphate buffered saline-Methylene Blue (PBS-MB) solution in a total volume of 10 µl. Five µl of antibody or 1X PBS-MB (negative control) and 1µl of neat or diluted sperm suspension were added to each well, covered and incubated for two hours at 37 ºC. The slides were then observed microscopically at 200 or 400 X magnification. Agglutination results were scored as follows: +++, complete agglutination, ++, large agglutinates, +, scattered agglutinates, -, no agglutination.

Dynabead FlowComp Flexi magnetic bead process

Antibody labeling was performed using the DSB-X Biotin Protein Labeling kit included in the Dynabead FlowComp Flexi magnetic bead kit from Invitrogen/Life Technologies, Carlsbad, CA, USA. 100µL of a 0.5 - 1µg/µL antibody solution and 50µL of freshly prepared 1M sodium bicarbonate solution were added to a 2mL reaction tube containing a stir bar provided in the kit. 200µL of dimethyl sulfoxide (DMSO, component B of the kit) were added to the kit's vial of DSB-X biotin succinimidyl ester (component A) and 6µL of this DSB-X biotin succinimidyl mixture was immediately added to the antibody solution. The reaction was allowed to stir at room temperature for 1.5 hours. The labeled antibodies were purified from unincorporated biotin using the spin columns and purification resin provided in the kit following the manufacturer's instructions. Based on the manufacturer's efficiency estimates antibody concentrations were calculated as follows: mg/mL DSB-X biotin-labeled protein = initial mg of protein x 0.85/mL collection volume.

Alternatively, antibody labeling was performed with the EZ-Link Sulfo-NHS-LC-Biotin kit from Pierce/Thermoscientific Rockford, IL. MOSPD3 was incubated with a 30 fold molar excess of biotin (11.5µL of 10mM freshly prepared biotin reagent to 100µL or 100µg of antibody), a 50 fold excess was used for monoclonal Ropn1l (5.2µL of 10mM biotin reagent to 100µL or 27.4µg of antibody) and a 20 fold molar excess of biotin was used for the polyclonal Ropn1l preparation (27.5µL of 10mM biotin reagent to 100µL or 180µg of antibody). Following Li et al (12) the reaction was set for 72 hours at 4^{o}C. Excess biotin reagent was removed via dialysis in potassium and amine free BupH PBS (phosphate buffered saline: 0.1M sodium phosphate, 0.15M NaCl, pH 7.2) Pierce/Thermoscientific Rockford, IL and using Slide-A-Lyzer Dialysis cassettes with a 10k molecular weight cut off also from Pierce. Again, following Li et al (12) dialysis was performed for 24 hours at 4^{o}C with two buffer changes.

Biotin incorporation or mole to mole ratio was measured using the Pierce Biotin Quantitation kit, now sold by Pierce/Thermoscientific Rockford, IL. The kit measures the change of absorbance at 500nm that occurs when the HABA (4'-hydroxyazobenzene-2-carboxyl acid) – avidin complex provided in the kit is disrupted in the presence of biotin. A biotinylated horseradish peroxidase (1mg/mL) included in the kit serves as the positive control. To preserve material, all antibodies were tested in a 1:10 dilution. Following the kit instructions HABA/avidin premix tubes were equilibrated to room temperature and resuspended in 100µL of ultrapure water. For each sample and the positive control a 1mL cuvette was filled with 800µL of BupH PBS, 100µL of HABA/avidin premix was added and mixed well. The A500 HBA/avidin absorption was measured at 500nm. Afterwards 100µL of the neat positive control and 1:10 dilutions for the positive control and labeled antibodies were added to each cuvette and measured at 500nm. The Pierce online calculator incorporates molecular weights and sample concentrations to translate the two absorption measurements into a ratio of biotin molecules per protein molecule.

For the magnetic bead capture 1µL of neat and 1:10 dilutions of sperm (representing 4,000 - 40,000 cells) were incubated with 10µL (5µg) DSB-X biotinylated antibody at 37°C on an Eppendorf Thermomixer at 800rpm for 1 hour. For the EZ-Link Sulfo-NHS-LC-Biotin labeled antibody the initial experiment with MOSPD3 employed three different amounts (10, 1.5 and 0.5µg) while the remaining experiments used 5µg of antibody as before. After incubation, unattached antibody was removed by adding 100µL of cold isolation buffer (phosphate buffered saline with 0.1% bovine serum albumin and 2mM Ethylenediaminetetraacetic acid -EDTA), brief mixing, pelleting the sperm at 6,000g for 10 min and removing the supernatant. The sperm pellet was resuspended in 100µL of cold Isolation Buffer and 10µL of FlowComp Dynabeads and incubated for 30 min at 4°C under constant motion on the Life Technologies HulaMixer. After incubation 100µL of isolation buffer were added and the samples were placed on a six tube DYNAL MPC-S magnet to immobilize the beads. The supernatant containing the non-target cells was removed and saved for extraction (Supernatant Fraction). The magnetic beads were resuspended in isolation buffer and immobilized twice more for a total of three washes. For the

final step, the magnetic beads were suspended in 200µL release buffer (provided in FlowComp Flexi kit), incubated for 30 min at 4°C on the HulaMixer and placed on the magnet. The release buffer now containing the captured cells was transferred to a fresh tube and stored for DNA extraction (Capture Fraction).

Protein G Magnetic Bead captures

1µl of sperm dilution (20-20,000 cells) was mixed with 5µl of unlabeled antibody (concentrations ranging from 0.1 to 1µg/µL) and incubated in an Eppendorf thermomixer for 2hrs at 37°C. After incubation the volume was adjusted to 50µl by adding 1X phosphate buffered saline (PBS). 25µl of resuspended Protein G beads (New England Biolabs, Ipswich, MA, USA) were mixed with 80µl of binding buffer ((0.1M sodium phosphate buffer pH 8.0) and the 50µl of sperm-antibody complex and incubated the sample at 4°C with 550rpm agitation for 30min. For phase III of the project this step was performed using the Life Technologies Hulamixer allowing for multidirectional mixing rather than horizontal agitation. After incubation the samples were placed on a DYNAL MPC-S magnet to immobilize the beads. The supernatant containing the non-target cells was removed and saved for extraction (Supernatant Fraction). The magnetic beads were resuspended in 500µl of binding buffer and immobilized twice more for a total of three washes. The beads were the stored in 20µL of 1X PBS prior to extraction (Capture Fraction).

Chelex extraction

Addition of Chelex beads and 1M DTT was modified based on the cell type and starting volume of the cell fractions as follows.

- Sperm cell supernatant and Dynal FlowComp Flexi capture fractions (starting volume 180-210µL): 20µL of 20% Chelex (Bio-Rad Laboratories, Hercules, CA), 50µL of sterile bidestilled water, 1µL of Proteinase K (20mg/mL) and 7µL of DTT (1M) were added to each sample.
- Sperm cell Protein G bead capture fractions (starting volume 20µL): 200µL of 5% Chelex, 1µL of Proteinase K (20mg/mL) and 7µL of DTT (1M) were added to each sample.
- Female cell supernatant and Dynal FlowComp Flexi capture fractions (starting volume 180-210µL): 20µL of 20% Chelex, 50µL of sterile bidestilled water, and 1µL of Proteinase K (20mg/mL) were added to each sample.
- Female cell Protein G bead capture fractions (starting volume 20µL): 200µL of 5% Chelex and1µL of Proteinase K (20mg/mL) were added to each sample.

All samples were incubated for 45min in a 56°C thermomixer. After incubation samples were vortexed for 10sec, then centrifuged for 15sec at 14,000g, incubated at 100°C for 8min, vortexed again, centrifuged for 3min at 14,000g, and stored at 4°C.

Amicon Filter Concentration

Amicon 50k Ultra-0.5 devices (EMD Millipore, Billerica, MA, USA) were pre-rinsed with 100µL TE-4 buffer. Samples were added and centrifuged at 14,000g for 8min. The reversed spin was 2min at 1,000g to transfer the concentrated sample from the device to a clean sample collection tube. Collection volumes ranged from 20-25µL.

QIAamp DNA Mini Kit DNA Extraction

20µL of Proteinase K (2omg/mL) and 200µL of sample were mixed with 200µL Qiagen Buffer AL (part of QIAamp DNA Mini kit; QIAGEN, Germantown, MD, USA) and 18.3µL of 1M DTT and incubated at 56˚C for 20min. Samples were cooled to room temperature and 200µL ethanol (96-100%) was added. Samples were added to the QIAamp Mini spin column (in a 2mL collection tube), and centrifuged at 6,000g for 1 min. The QIAamp Mini spin column was placed in a clean 2mL collection and after adding 500µL Qiagen Buffer AW1 centrifuged at 6,000g for 1 min. This step was repeated with 500µL Qiagen Buffer AW2. DNA was recovered by placing the QIAamp Mini spin column in a clean 1.5mL microcentrifuge tube and adding 200µL Qiagen Buffer AE, incubating at room temperature (15-25˚C) for 1 min, and then centrifuging at 6,000g for 1 min. All samples were concentrated using Amicon 50k Ultra devices as described above.

Process controls

The following controls were added to the experimental set-up.

Agglutination test:

- Negative control: semen dilutions were prepared and incubated as described above without adding any antibody.

Magnetic bead capture:

- Negative control: all reagents including antibodies were subjected to the full procedure with all wash steps, extraction and quantitation without adding any human cells.
- No antibody control: biological test samples were subjected to the full procedure with all wash steps, extraction and quantitation with 1x PBS replacing the antibody volume in the initial antibody binding reaction.
- Positive full process control: corresponding cell counts for each donor were incubated in the same buffer, with the same antibody and identical conditions parallel to the bead binding step, but without beads.

DNA extraction:

- Positive “extraction only” control: corresponding cell counts for each donor were extracted together with the test sample fractions without undergoing the bead incubation step.

STR typing:

- Positive control: control DNA provided by the manufacturer was typed in parallel to the samples.

- Contamination control: reference samples from project workers were typed to test for contamination.
- Amplification negative control: all amplification reagents were amplified and typed without DNA addition.

DNA Quantitation

Male and total DNA in all DNA extracts was quantitated using the Quantifiler Duo® DNA Quantification kit (Applied Biosystems - Lifetechnologies, Foster City, CA) for Real-Time (RT) PCR Model 7500 according to the manufacturer's protocol.

STR Typing

For phase 1 and 2 samples, references and other controls with quantitation results within the recommended range of input DNA for the AmpFlSTR® Identifiler Plus Kit (Applied Biosystems - Lifetechnologies, Foster City, CA) were amplified on the Gene Amp PCR 9700 System (Applied Biosystems) and typed on the 310 Genetic Analyzer (Applied Biosystems – Lifetechnologies, Foster City, CA) according to the manufacturer's instructions. DNA profiles were determined using the GeneMapper® *ID* (Version 3.2) software package from Applied Biosystems with the analytical threshold set to 50RFU. According to the Identifiler Plus Manual the recommended DNA input is 0.5-1.25 ng, and 10µl of extract can be added to the PCR reaction mix, therefore all extracts yielding 0.05- 0.125 ng/µl DNA were candidates for STR amplification. Resulting profiles were compared to the buccal reference swabs and contamination control samples.

Results

Agglutination assay

The original batch of antibodies obtained from Abnova, Taipei, Taiwan that was tested included: SPAM1 (polyclonal), MFGE8/SEDI (polyclonal and monoclonal), ADAM2 (polyclonal and monoclonal), and UBAP2L (polyclonal). Most of these showed only weak scattered agglutination results for the neat antibody addition and no agglutination for any dilutions. The exceptions were SPAM1 which showed no agglutination at all, and ADAM2 (monoclonal) showing some scattered agglutination for the neat and 1:2 and 1:4 antibody dilutions, ADAM2 polyclonal reacting for neat and 1:2. The same titration for AKAP3 (polyclonal) obtained from Antibodies-Online Inc, Atlanta, GA, USA resulted in large agglutinates for the 1:4 dilution and scattered aggregates were seen down to the 1:256 antibody dilution. An attempt to repeat this agglutination test for the additional semen samples acquired in phase 3 was inconclusive due to the presence of undefined clumps of material and aggregates resembling agglutination in the negative controls.

The results for the MOSPD3 antibody ordered from Abcam, Cambridge, MA were positive for sperm-antibody binding. Large agglutinates were seen for the neat and1:4 samples. This titration series stopped at 1:8 and this final dilution showed scattered agglutination.

Magnetic bead capture results

Phase 1 – full seven antibody panel - Protein G beads
Experiments were performed as described above using two semen donors (01s and 02S) and five different sperm counts (20,000; 2,000; 200; 20 and 2). Two positive controls (one for each semen source) were processed using the same cell counts. Extraction yields for the combined capture and supernatant fractions were similar for the positive control and correlated well with the cell count. For cell counts below 2,000 most of the DNA concentrations were below the quantitation assay threshold which was why the Amicon concentration step was added to the procedure. Table 2 shows the ratio between the DNA concentrations in the bead capture fraction versus the supernatant or first wash fraction. Each value represents a single sample. Ratios above 2 are marked. These experiments did not include a no-antibody sperm capture control. Based on these results, the four antibodies with an increased amount of DNA in the bead fraction versus the supernatant fraction (UBAP2L, MFGE8m, ADAM2m and AKAP3) were selected for further testing.

Table 2 Calculated ratios between DNA concentrations in capture versus supernatant fraction for the 7-Antibody panel and Protein G beads,

	SPAM	UBAP2L	MFGE8m	MFGE8p	ADAM2m	ADAM2p	AKAP3
01S 20k	0.85	2.47	2.18	0.46	2.65	0.74	2.57
01S 2k	1.07	3.29	1.99	0.24	4.34	0.15	1.47
02S 20k	1.09	2.78	3.83	0.18	2.66	0.20	0.66
02S 2K	1.13	0.17	3.59	0.19	2.64	0.29	1.43
nc 01S 20k	0.99	5.40	7.68	1.40	3.95	0.68	5.42
nc 01S 2k	1.30	0.70	35.47	1.34	und	0.24	7.92
nc 02S 20k	2.05	7.86	6.02	0.24	2.94	0.75	2.95
nc 01S 2K	4.39	und	24.50	0.46	und	1.69	10.53
ratio >2	2	5	7	0	6	0	5

"und" indicates no quantitation reading obtained for one of the fractions.
"nc" not concentrated

DNA typing of eligible DNA fractions resulted in the expected partial to full single source profiles. The number of loci detected strongly correlated to the initial DNA input (data not shown).

Phase 2 – Reduced four antibody panel - Protein G beads
Subsequent testing included only the four most promising antibodies and omitted the 200 and 20 cell samples. This means there were now three cell count titration steps for each semen source (20,000; 2,000 and 200). In this phase the Amicon Ultra 50k filter concentration step was added to all Chelex extractions. Other modifications included adding the "no antibody control" and doubling the amount of antibody added to the reaction. Samples were tested in parallel using 5uL and 10uL of antibody. Overall yields for this set of experiments were low. The positive controls yielded between 33 and 86% of the DNA amount expected based on the cell count. For both test samples magnetic bead capture and supernatant fractions combined, only recovered approximately 16% of the expected DNA.

As depicted in table 3, for the ADAM2 antibody the bead and wash fractions did not show the expected enrichment in the bead fraction seen in the first round of testing.

Table 3: Quantitation values of cells captured in the sperm-ADAM2 (monoclonal)-bead complex (in pg/µL)

	Beads - 01S	Wash - 01S	Beads - 02S	Wash - 02S
No Ab 20K	**37**	**15**	**69**	**3**
No Ab 2K	2	7	6	1
No Ab 200	0	0	0	0
5 uL Ab 20K	27	22	100	117
5 uL Ab 2K	6	8	18	6
5 uL Ab 200	1	1	1	0
10 uL Ab 20K	15	196	92	143
10 uL Ab 2K	2	5	5	8
10 uL Ab 200	0	1	0	1

For the MFGE8 antibody only two of the 20K samples for donor 01S showed any enrichment in the bead fraction (see highlights), but overall yields were low, the result was not confirmed for donor 02S, and the no antibody control also showed more DNA in the bead versus the wash fraction (table 4).

Table 4: Quantitation values of cells captured in the sperm-MFGE8 (monoclonal)-bead complex (in pg/µL)

	Beads - 01S	Wash - 01S	Beads - 02S	Wash - 02S
No Ab 20K	**37**	**15**	**69**	**3**
No Ab 2K	2	7	6	1
No Ab 200	0	0	0	0
5 uL Ab 20K	31	3	100	96
5 uL Ab 2K	2	23	5	6
5 uL Ab 200	1	0	0	0
10 uL Ab 20K	77	11	61	125
10 uL Ab 2K	5	11	10	7
10 uL Ab 200	1	0	0	0

Table 5 shows results for the UBAP2L antibody. Two of the 20K samples for donor 01S and one 2k sample for donor 02S (see highlights), but none of the other samples with the exception of the same “no antibody” control showed any enrichment in the bead fraction. Again overall yields were low.

Table 5: Quantitation values of cells captured in the sperm-UBAP2L (polyclonal)-bead complex (in pg/µL)

	Beads - 01S	Wash - 01S	Beads - 02S	Wash - 02S
No Ab 20K	**37**	**15**	**69**	**3**
No Ab 2K	2	7	6	1
No Ab 200	0	0	0	0
5 uL Ab 20K	22	11	130	194
5 uL Ab 2K	9	21	6	3
5 uL Ab 200	0	0	0	0
10 uL Ab 20K	89	17	91	147
10 uL Ab 2K	4	3	11	1
10 uL Ab 200	0	0	0	0

The 5uL and 10ul amounts of antibody were very similar and did not show a trend; these results were therefore combined (n=2) to calculate the average capture versus supernatant yield ratio.

Table 6: Average ratio between DNA concentrations in capture versus supernatant fraction: Protein G beads

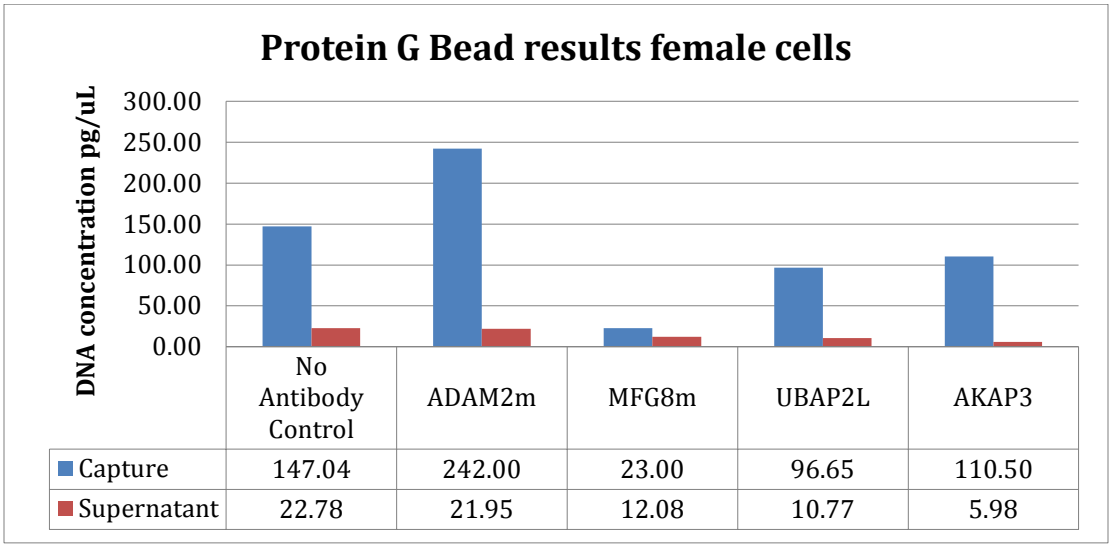
	UBAP2L*	MFGE8 m	ADAM2 m	No Antibody control
01S 20k	3.98	7.65	0.20	2.56
01S 2k	0.52	0.22	0.63	0.27
02S 20k	0.65	0.73	0.74	25.75
02S 2K	5.17	1.13	1.65	5.65

* ratios >2 are highlighted

Clearly the bead fraction captures DNA independent of the presence of antibodies in the assay. The experiment testing the AKAP antibody was inconclusive. This run using semen donors 2 and 3 had been modified by adding 1 hour of incubation of the magnetic bead fraction at 37°C prior to the Chelex extraction. Here all ratios between capture and supernatant fraction, including for the "no antibody" control, were below 1 signifying less capture by the Protein G beads. But all DNA yields for all fractions were very low indicating a problem with this extraction, possibly caused by degraded DTT (data not shown).

The unspecific binding of the protein G beads was confirmed in the experiments using female cells. Two independent runs with defined cell counts of 400 vaginal epithelial cells both resulted in more DNA in the bead capture fraction. For the second run testing either 5µL or 10µL of each

antibody, the positive extraction control yielded much more than the expected 2.4ng total yield, which indicates problems with adding defined cell counts (data not shown). As seen in the graph in Figure 1, there are also variations in the overall DNA amounts recovered for this sample donor. Again, results for 5 and 10µL antibody tests were combined so n=2. Figure 1 also shows that all of the bead capture fractions contain epithelial cell DNA, which confirms the unspecific binding seen for the sperm cell experiments.



	No Antibody Control	ADAM2m	MFG8m	UBAP2L	AKAP3
Capture	147.04	242.00	23.00	96.65	110.50
Supernatant	22.78	21.95	12.08	10.77	5.98

Figure 1: Average DNA concentrations in pg/µL for bead capture and supernatant fractions for female samples after incubation with four semen specific antibodies.

Again, DNA typing of eligible DNA fractions resulted in the expected single source male or female DNA profiles (data not shown). Profile quality varied with samples with lower DNA yields showing partial types.

This set of cell capture results raised several issues: for all sperm cell experiments DNA yields, even when combining the two fractions, were generally very low with often less than 15% recovery of the theoretical DNA contained in the defined cell amounts. Then there was the non-specific binding of cells or DNA to the Protein G beads that could be shown for both the female cells and the "no antibody" controls for the sperm and epithelial cell experiments. While silica coated beads are routinely used for DNA capture and extraction, Protein G covered beads are very specific to mouse or rabbit immunoglobulin (6) and should not have captured intact vaginal cells or, if lysis is assumed, any nuclei or chromosomal DNA. In order to troubleshoot these results, these Protein G bead experiments were repeated with fresh biological samples and the "extraction only" control was introduced.

Phase 3 –Protein G beads result confirmation with fresh biological samples
The first set of three independent experiments involved 1µL of fresh semen and 5µg of antibody

MFGE8. Instead of using normalized cell counts, a positive control for each donor was processed in parallel (without beads) to serve as a benchmark for the expected yield. Sperm counts for the purchased semen samples M1 and M2 had been determined to be 40,000 and 17,000 sperm per µL. With the exception of using the multidirectional mixer there were no changes to the previous protocol. The ratio between the bead fraction and the cells remaining in the supernatant for both donors resulted in an average of 17.4 (n=6). Protein G beads again captured sperm cell DNA. All of these experiments unfortunately did not include a "no antibody" control, so this data set in itself cannot be used to evaluate unspecific binding.

Capture fraction DNA recovery was slightly higher than the positive control recovery, but overall yields including the "complete process positive control" again were low compared to the known sperm count. In order to investigate the low yields, a second set of positive controls was added to the Chelex extraction set. These fresh samples were not subjected to the two lengthy incubation and multiple bead wash steps necessary for the bead separation. The DNA yield for the first experiment using "extraction only" controls, the DNA recovery in the these controls was ten times as high as for the other samples (data not shown).

Figure 2 shows the results for an additional Protein G bead experiment employing both semen donors, both antibodies, and again the "extraction only" positive controls. Each bar represents a single sample.

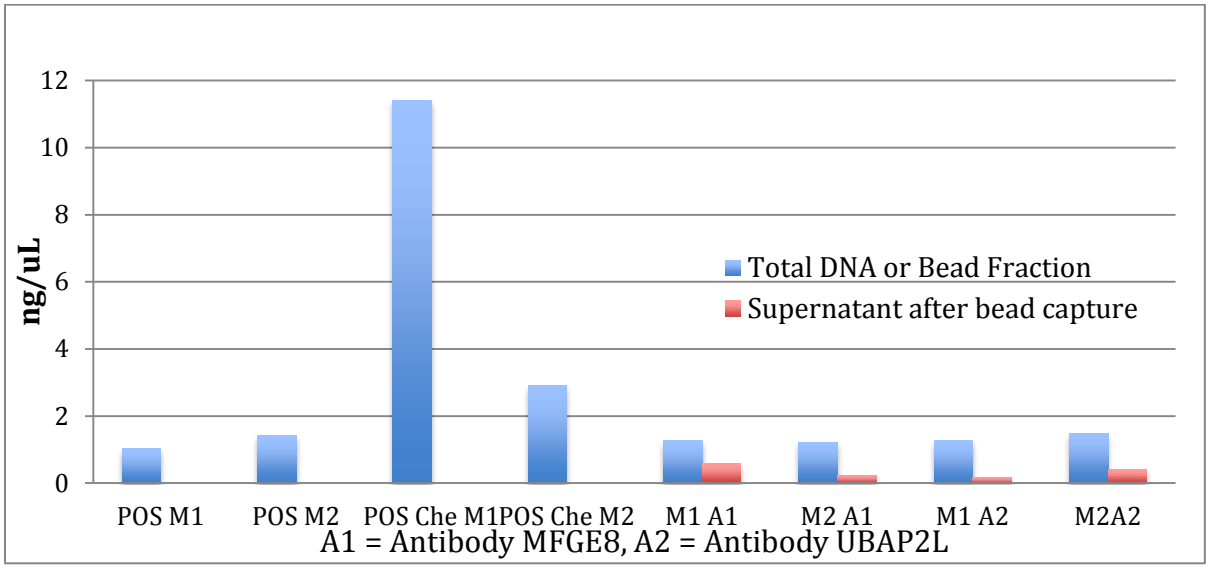


Figure 2: Human DNA quantitation results for positive controls and two semen donors (M1 and M2) after Protein G bead procedure; DNA concentrations in ng/µL for bead and supernatant fractions after incubation with two semen specific antibodies; "POS Che" indicates the two extraction only controls added to the Chelex extraction set after the incubation steps.

The amount of DNA in the bead/capture fractions is similar to the two positive controls that were

incubated with the antibodies and processed in parallel with no beads added. There is less DNA in the supernatant. The "extraction only" controls vary in yield, but the >10ng/µL concentration seen for donor M1 is very close to the yield in the previous experiment mentioned above and it is unlikely that this ten-fold difference in DNA recovery is caused by pipetting variation. With the regular positive controls, which do not contain any beads, showing the same loss of DNA, the yield reduction cannot be caused by the magnetic beads present in the semen samples. A quantitation artifact is also excluded as the cause. All samples and controls were concentrated using Amicon Ultra filter membranes; a step which normally is also efficient at removing PCR inhibitors. The Quantifiler Duo Internal Positive Control (IPC) and the sample curve slopes did not indicate any type of inhibition for the regular positive controls and the samples. The only remaining option is that one or both of the necessary incubation steps (2h at 37°C with the antibody and 30min at 4°C with the beads) cause a certain amount of cellular and DNA degradation which leads to a loss in DNA yield.

The single source cell type Protein G experiment was also repeated for vaginal epithelial cells. Cells were eluted from the swab and small aliquots from the cell pellet and the elution fraction were used for the capture/extraction procedure. As before, an equivalent positive control was utilized to replace the cell counting step. Two experiments were performed for MFGE8. Figure 3 displays the results for the experiment employing both antibodies, the initial MFGE8 results were very similar. Both the pellet (labeled F1A) and the elution buffer fraction (labeled F1B) yielded only low amounts of DNA with the pellet being more concentrated. Again, the "extraction only" positive control has a much higher yield. For the magnetic bead samples almost the entire DNA is in the bead capture fraction which confirms that there is clearly an affinity of Protein G beads to human cells (or possibly DNA) that is not related to the presence of specific antibodies.

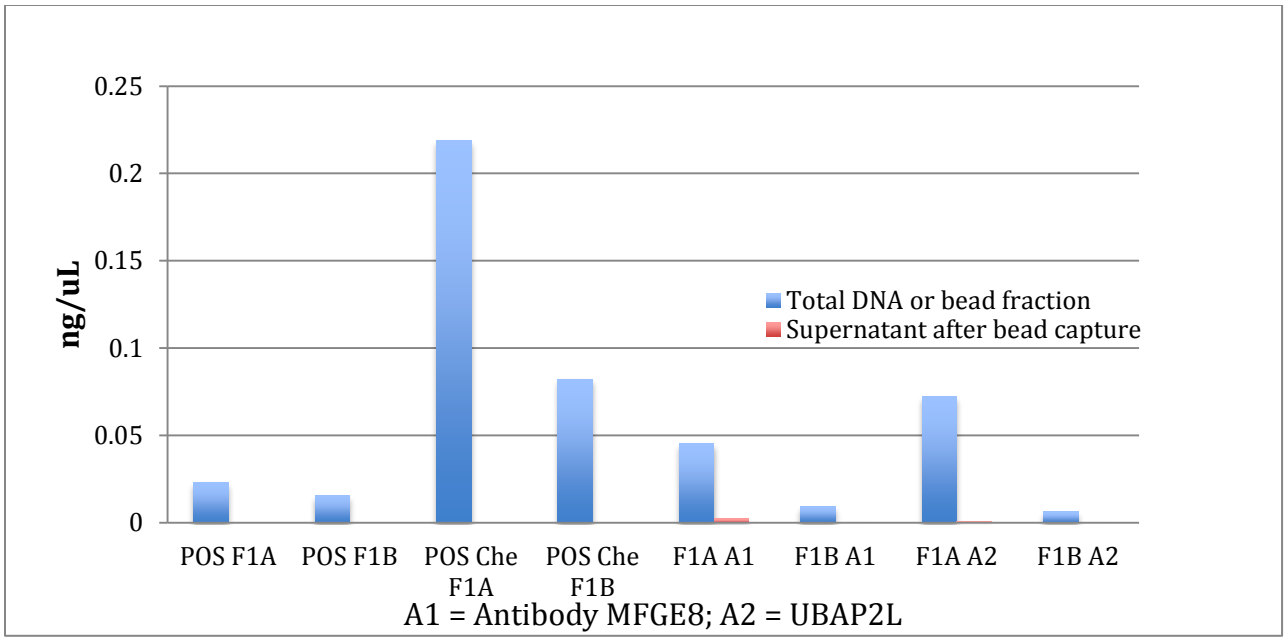


Figure 3: Human DNA quantitation results for positive controls and two amounts of female cells (F1A and F1B) after Protein G bead procedure; DNA concentrations in ng/µL for bead and supernatant fractions after incubation with two semen specific antibodies; "POS Che" indicates the two controls added to the Chelex extraction set after the incubation steps; each bar represents a single sample.

Protein G Magnetic beads as purchased from New England Biolabs are described as nonporous paramagnetic particle covalently coupled to a truncated form of recombinant Protein G. It is unclear what exactly causes the unspecific binding and how the surface could be blocked to only allow for specific IgG – Protein G reactions. The New England Biolabs protocol for Protein G beads, is geared towards the isolation of immunoglobulins and antibody facilitated immunoprecipitation of target proteins out of crude cell lysates (6). Similar Pan Mouse IgG coated beads are available from Lifetechnologies and marketed as a tool to either positively isolate or deplete antibody bound target cells (50). Anslinger et al (11) employed these IgG beads in their research and also observed unspecific binding and co-extraction of female cells for their mixtures of buccal cells and varying amounts of spermatozoa.

Theoretically, the assay described here is employing intact cells and, especially for the male compact sperm cells with membrane containing strong disulfide bonds, there should have been no cell lysis until the Chelex extraction. On the other hand, the higher DNA yield for the "extraction only" control indicates possible cell and DNA degradation prior to the extraction step. It is possible that multidimensional agitation in the presence of metallic beads could have led to physical rupture of cell membranes.

Phase 4 – Dynabead FlowComp Flexi magnetic bead capture

Li et al (12) describe the successful enrichment of sperm cell DNA from fresh single source and mixed semen samples, as well as dried vaginal swabs using the Dynabeads FlowComp Flexi kit from Invitrogen. In this project the first sets of phase 4 experiments using this kit, labeled MFGE8 antibody and both semen and vaginal cell samples, yielded some DNA in the bead capture fractions, but all positive controls and the supernatant fractions were negative for human or male DNA. This was due to real time PCR inhibition. The Quantifiler Duo Internal Positive Control (IPC) designed to indicate PCR inhibition only amplified in sample fractions recovered in release buffer (capture fractions). All other samples including the positive controls, which prior to the addition of the Chelex beads were still suspended in incubation buffer, showed complete amplification failure with no signal for the IPC.

One component of the isolation buffer must have been co-purified during the Amicon Ultra 0.5 concentration step and caused this real time PCR inhibition. The isolation buffer as described by the manufacturer consists of phosphate buffered saline (PBS) that is supplemented with 0.1% bovine serum albumin (BSA) and 2mM ethylenediaminetetraacetic acid (EDTA). EDTA is a known PCR inhibitor and may not have been removed by the 50k NMWL (nominal molecular weight limit) cut-off membrane. Clearly the Dynabead FlowComp Flexi isolation buffer was incompatible with the Chelex single tube extraction even after Amicon filtration; therefore for future experiments the downstream DNA purification method was replaced by a silica column based DNA extraction method (QIAamp DNA Minikit from Qiagen).

There are three major differences between the Protein G bead assay as performed for this grant and the Dynabead FlowComp Flexi magnetic bead capture as described by the manufacturer and Li et al (12). The antibody binds to the beads via a biotin-streptavidin intermediate, the assay contains a sperm pelleting step designed to remove unincorporated antibody and instead of adding the bead/cell conjugate to DNA extraction, cells are separated from the beads with release buffer incubation. All of these steps are designed to make the assay more specific but may also result in a certain loss of cellular material.

Five subsequent magnetic bead capture experiments with four biotin labeled antibodies and either single source sperm or vaginal epithelial cells failed to show the expected sperm DNA capture. All resulting QIAamp DNA fractions were successfully quantitated but none of the bead fractions, including the samples containing sperm and labeled antibodies, indicated any DNA enrichment. The resulting DNA yields were comparable to the positive controls but resided almost exclusively in the supernatant fraction. While some of the experiments included slight variations in incubation conditions and number of sperm cells used, all results were similarly low for the bead capture fraction and were pooled (sample numbers range from one to six) to calculate the ratios shown in table 7.

Table 7 Average ratios between DNA concentrations in capture versus supernatant fraction: Dynabead FlowComp Flexi

Cell type	Antibody	Average Ratio
Sperm	MFGE8	0.22
	UBAP2L	0.01*
	MOSPD3	0.04
	AKAP3	0.17
	No-antibody controls	0.24
Vaginal	MOSPD3	0.08
	No-antibody controls	0.10

* This value is not an average but a single measurement.

For two of the MOSPD3 experiments we microscopically examined the magnetic bead fraction after completion of the three wash steps. No adhering sperm cells were seen. It is unclear if this failure of sperm capture is based on lack of specificity of the antibody, problems with the biotin labeling step or other buffer/temperature incompatibilities.

As a final protocol modification, new batches of antibody for MOSPD3 and, since AKAP3 was not available from Abcam, AKAP associated protein or Rpn1l (polyconal and monoclonal) were tested using the biotinylation assay employed by Li et al (12). The biotin quantitation assay gave the expected result for the neat positive control, but showed a different result for the 1:10 dilution of the positive control and less than one biotin molecule per immunoglobulin G for the 1:10 dilution of the antibodies. Possibly the 1:10 dilutions were below the detection limit for the assay. Instead of 100µg as for the neat positive control, only 2.7 to 20µg could be added for each antibody, otherwise the samples would have been consumed. Magnetic capture was tested for three different input amounts was tested for MOSPD3, but no capture fraction enrichment was seen. To possibly remedy the low incorporation rate, all three already labeled antibodies were then subjected to another round of labeling followed by biotin quantitation. There was no improvement in the quantitation results. Regardless of the biotin quantitation result, all three labelled antibodies were then tested in a magnetic bead capture experiment with 5µg of each antibody. As can be seen in Figure 4, again almost the entire DNA resided in the supernatant fraction.

For MOSPD3 microscopic examination of the magnetic bead fraction revealed a single isolated observation of a sperm head and bead next to each other, but this could have been accidental and does not prove an interaction.

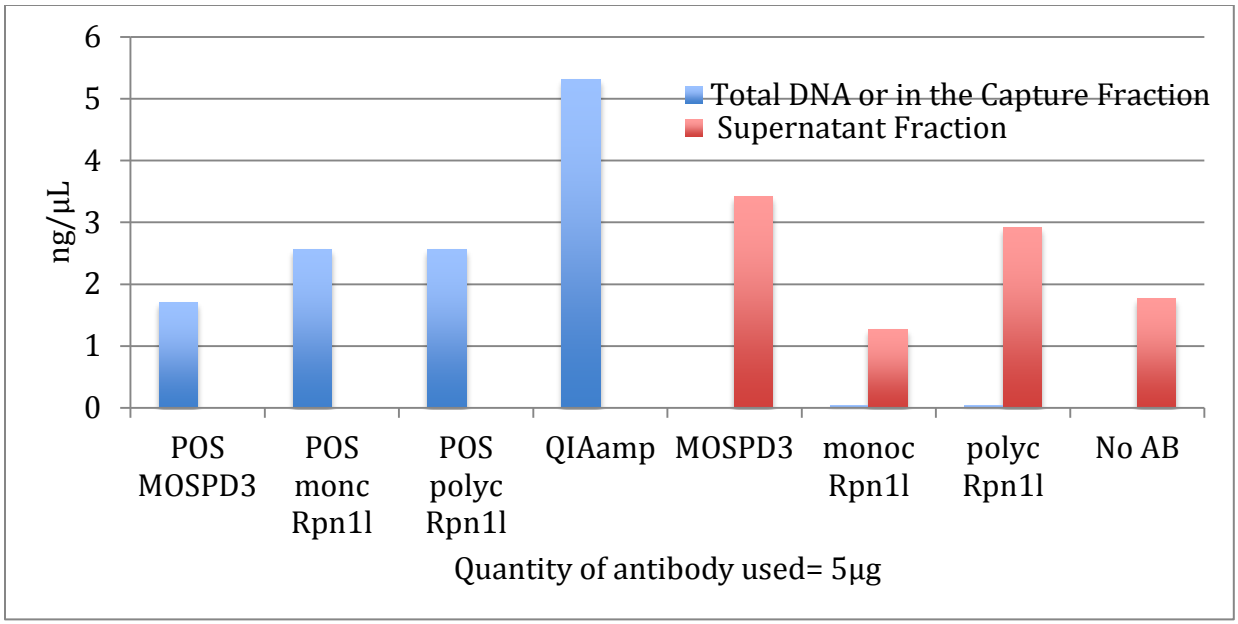


Figure 4: Human DNA quantitation results for samples after EZ-Link Biotinylation and controls; DNA concentrations in ng/µL for bead and supernatant fractions after incubation with three semen specific antibodies; "QIAamp" indicates the "extraction only" control added to the Qiagen extraction set after the incubation steps; each bar represents a single sample.

With the lack of success for single source sperm samples the project never reached the stage of testing cell mixtures and dried semen stains.

Discussion

This project failed to demonstrate sperm cell specific enrichment for either magnetic bead capture assay. Protein G beads were shown to retain DNA, but in a non-specific fashion including vaginal epithelial cells and control samples with no antibody addition. For the Dynabead FlowComp Flexi kit almost the entire recovered DNA resided in the supernatant fraction.

Developing a working magnetic based sperm capture assay has been an elusive goal over many years. Early data produced by Eisenberg (9, 10) and Anslinger et al (11) were never published in a peer review journal and neither team has pursued this topic with more research. This lack of progress cannot have been due to the scarcity of known antibodies. Clinical research concerned with human fertility has thoroughly explored sperm surface characteristics and identified many possible antigens (53, 54). This number of available target sites has grown exponentially with the advent of proteomics research and proteomics databases (55, 56). Magnetic beads have also evolved to be more user friendly and easy to use products. The Invitrogen Dynabead FlowComp Flexi kit tested in this study and other Dynabead products are now routinely being used for a variety of cell enrichment applications (for example 2-4). While it has to be noted that many of

the beads are designed to work with whole blood or cell cultures, and normally require an excess of fresh material, it is also true that these assays can be made very specific and that the beads successfully retain the target cells (7). Clearly antibody based magnetic bead capture should work at least for fresh sperm cells and this was demonstrated for three different types of beads with different coating (10-12). The success rate declines for dried specimen, especially with increasing age. Older semen evidence mostly contains sperm heads and no intact spermatozoa with the tails attached, which means antibodies binding to the tailpiece will lose effectiveness (11, 12). Another reason for the loss of recovery could be membrane degradation and loss of antigen specific quaternary structure and binding capacity.

For the Protein G beads investigated here the problem was not a lack of DNA recovery but rather the unspecific retention of DNA from sperm and non-sperm cells, with and without the addition of sperm membrane receptor specific antibodies. It is unlikely that the beads were binding whole cells. The microscopic examination of a Protein G sperm extraction bead fraction after bead immobilization and washing did not reveal any cell to bead adhesion. While there has been some discussion on cell free DNA (21), it is more likely that cells were lysed during the magnetic bead incubation step. For most experiments cells were not frozen prior to use and should have been intact after suspension in phosphate buffered saline, but the sodium phosphate Protein G bead binding buffer combined with mechanical mixing in the presence of metal beads may have disrupted not only epithelial cell but also sperm head membranes. These magnetic beads may have an affinity to DNA or DNA-histone complexes either via the Protein G coating or possibly gaps in this coat. No experiments with cell lysates or previously extracted DNA were performed to explore this.

Rather than spending more time on the Protein G magnetic bead type, the team decided to explore the Dynabead FlowComp Flexi kit as described by Li et al (12). Antibodies tested were the four selected for this study and the MOSPD3 antibody employed by Li et al (12). None of the antibodies produced any enrichment in the bead capture fraction. There are three possible areas where this assay can fail: the biotin labeling reaction could be unsuccessful leading to unlabeled and thus ineffective antibodies. The labeling chemistry used in this study was DSB-X biotin succinimidyl ester, part of the Dynabead FlowComp Flexi kit and was run for 1.5 hours. Li et al (12) deviated in this step and labeled the MOSPD3 antibody with sulfo-NHS-LC-Biotin obtained from Pierce Protein Biology Products, Thermo Scientific, Rockford, IL, USA; also the labeling reaction was allowed to incubate for 72 hours. Theoretically the labeling should not have been an issue. Invitrogen technical support had been consulted on the technical details and, for example, Jensen et al (2) used the same chemistry. The company specifically warns against certain antibody storage buffer additives that may interfere with antibody labeling and may require prior dialysis. In this project these additives were either not present or below the problematic threshold. On the other hand the reaction was performed multiple times with different antibodies without producing any successful DNA capture. Also the actual binding of the biotin label was

not confirmed via Western blot and labeled streptavidin probes, so a systemic problem in the labeling step could not be excluded. To address this possible source of failure, MOSPD3 and Ropn1l were labeled following Li et al (12) as closely as possible and the labeling efficiency was monitored using a biotin quantitation kit. The quantitation revealed low incorporation and the subsequent capturing experiments failed to show any enrichment. Antibody conjugation is not as straightforward as indicated in commercial kits and may require antibody specific optimization; in addition NHS chemistry has the potential of putting the label in the binding domain of the antibody and neutralizing activity (peer review comment). More work could be done to produce high density MOSPD3 biotin conjugates.

The other factor that would prevent any specific binding is the choice and the quality of the purchased antibodies. Most antibody manufacturers only test their antibody performance for Western Blot applications and researchers have encountered vast differences in quality and binding capability for the same type of antibody from different companies (Kappen, Dreses-Werringloer, personal communication). For the phase 1 antibody panel, only the AKAP3 antibody obtained from Antibodies Online, Atlanta, GA showed large agglutinates. All of the agglutination tests for the antibodies from Abnova, Taipei, Taiwan were either weak (scattered agglutination for undiluted antibody) or inconclusive (aggregates in negative control). It was unfortunately not possible to purchase the more promising antibodies from different sources and compare the specificity for different lot numbers and providers. The MOSPD3 antibody employed here was polyclonal, produced in rabbit and from a different company than the monoclonal MOSPD3 antibody used by Li et al (12). This antibody was also shown to produce large agglutinates. Even with high quality antibodies there is no guarantee that the sperm membrane antigen in its mature post ejaculation form will be recognized and many theoretically feasible antibodies will not show the expected reaction. For this project, at least the AKAP3 and MOSPD3 antibodies had been shown to react with sperm and should have facilitated sperm capture.

Under the assumption that antibodies were sufficiently labeled, sufficiently specific to an accessible sperm membrane antigen, and buffer and temperature conditions are suitable for efficient binding, the last factor prohibiting cell enrichment is the strength of the non-covalent biotin-streptavidin bond and its reaction to shearing effects during the required wash steps. As described by Eisenberg (10) agitation and stringent washing led to loss of previously bound material for the epoxy-antibody bead conjugates, which is why the stabilizing photolysis step was added. It remains a fact that many other research teams have successfully recovered their target cell population (2-4). Overall performance will also be affected by the density of antigens available on the target for binding, number of biotin molecules incorporated per antibody and density of streptavidin receptors on the beads. For many of these applications there is abundant starting material and a certain loss of cellular material is expected and tolerated. In this study starting amounts of sperm cells were fairly high, which makes it unlikely that that lack of

thermodynamic stability and physical disruption of already formed bonds is the explanation for the total failure to capture sperm cell DNA on the streptavidin coated beads.

Conclusions and implications for further research

This study clearly demonstrated that Protein G beads are not suitable for magnetic bead based sperm separation. For the streptavidin coated beads, the failure to duplicate the results described by Li et al (12) may be based on a combination of factors with antibody quality and some of the buffers and incubation conditions being possible factors. But at least for the EZ-Link sulfo-NHS-LC-biotin kit, reaction the most likely step to have failed in our hands was the antibody biotinylation, which means the work presented here was compromised by technical issues and cannot be used to decide on the overall scientific merit of the magnetic bead sperm capture method.

The paper by Li et al is the only peer reviewed publication on the successful recovery of sperm cells and male DNA from mixtures with female cells using streptavidin covered beads (12). But they also found that the assay was better suited for fresh sperm and describe their success rate declining rapidly for dried vaginal swabs after short storage periods. It is unclear, if further research would be able to improve on these results. Surface antigens are easily compromised and capture release assays are always accompanied by a certain amount of loss. Forensic evidence by nature does not consist of pristine and abundant cells, and even with very effective antibodies and under optimized conditions magnetic bead assays will not be able to recover the highest possible amount of male DNA. This makes the idea of an automated immuno-magnetic sperm capture assay problematic for compromised samples and evidence with low sperm content. If a working assay could be developed, its application is likely to be limited to fairly fresh and abundant samples.

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Dissemination

It is generally not possible to publish negative results, especially if the failure to demonstrate a hypothetical mechanism is mostly based on technical difficulties. This would be different, if we had been able to identify true scientific reasons why the tested streptavidin coated bead assay did not yield the predicted results. Unfortunately that was not the case, and with the exception of this final report, we are not planning to submit this material for publication.