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Automated sperm identification using MetaSystems Metafer imaging system

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Thesis

AUTOMATED SPERM IDENTIFICATION USING METASYSTEMS METAFER IMAGING SYSTEM

by

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ABSTRACT

Thousands of sexual assault cases in the United States are backlogged. This has been a growing issue for years that has increased the difficulty of solving these cases and providing closure to the victims. The analysis process for each case includes the identification of body fluids, presumptive testing, confirmatory testing, and DNA extraction. The only confirmatory method for semen identification is a microscopic visualization of sperm cells. The time spent on microscopic analysis varies depending on the complexity of the samples and the skills of the analyst. While the identification of sperm cells is informative, it can be very time-consuming and labor intensive. Some forensic laboratories choose to skip this step and submit samples directly for DNA analysis. Conducting DNA analysis on unscreened samples can increase the cost of testing when negative samples are analyzed as well as the time it takes to process each case.

Automated microscopy has been available for decades and more recently has been paired with artificial intelligence to detect sperm cells on microscope slides. In this research, the MetaSystems automated microscope was used to analyze slides that mimic forensic sexual assault samples. Slides were also examined using traditional microscopy. The automated system quickly provided an accurate quantification of the number of sperm cells present in a sample, which can inform downstream DNA testing. The software was successful in identifying sperm cells treated with Christmas tree and hematoxylin and eosin

stains, even among epithelial cells and various contaminants. Results demonstrated that an artificial intelligence-driven forensic sperm cell detection microscope can significantly reduce the time it takes to locate and identify sperm cells and estimate sperm cell quantity compared to a lengthier and more tedious manual search. Drawbacks to the system include the relatively high cost and reduced ability to accurately detect sperm cells amid contaminants that are of similar morphology.

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AI	Artificial Intelligence
ALS	Alternate Light Source
AP	Acid Phosphatase
DNA	Deoxyribonucleic acid
H&E	Hematoxylin and Eosin
KPIC	Kernechtrot Picroindigocarmine
PSA	Prostate Specific Antigen
SANE	Sexual Assault Nurse Examiner
Sg	Semenogelin

1. INTRODUCTION

1.1. Semen Identification in Casework

Evidence collected from crime scenes is routinely submitted to crime labs for further analysis. One of the main roles of a forensic biologist is to locate and identify body fluids such as blood, saliva, and semen (1). Locating body fluids increases the potential of detecting deoxyribonucleic acid (DNA), which may increase the likelihood of making associations between the victim, crime scene, and suspect (2).

In the United States, more than half of women and about one-third of men will have experienced sexual violence that involves physical contact (3). Sexual assault cases that are reported in hospitals are usually handled by Sexual Assault Nurse Examiners (SANE). A SANE is a registered nurse with specialized training that enables them to care for sexual assault victims as well as conduct forensic examinations to collect evidence for further analysis (4). SANEs are also trained to provide testimony in civil and criminal cases. Sexual assault evidence that is collected by a SANE or other investigators is subsequently submitted to a forensic laboratory for further examination.

The first step a forensic biologist performs when analyzing an item of evidence is a visual examination. The visual examination is conducted under white light to identify visible stains then, if necessary, an alternate light source (ALS) is used as a non-destructive and quick secondary technique. In most cases of sexual assault, the most probative

biological fluid is semen. Under white light, the color of semen stains can appear faint yellow or off-white. With stains that are not visible under white light, an ALS can be used to help identify and locate evidence such as saliva, semen, urine, and fibers. Using ALS at a wavelength of approximately 450 nm (blue light) causes many semen stains to fluoresce (5).

Once a suspected semen stain is located, testing is conducted to confirm the presence of semen since other body fluids and non-biological stains can also fluoresce. The components of semen are produced in four regions of the male reproductive tract. The first region is the testes, which produce spermatozoa before they are matured in the epididymis and contribute 5-10% of the total semen volume. The testes are also responsible for the production of inhibin and testosterone hormones. The second region is the seminal vesicles, which are responsible for approximately 65-75% of semen volume (6) and produce the fructose that is found in semen and used as an energy source for spermatozoa. The third region is the prostate gland, which produces 20-30% of the volume of semen. The fluids that originate from the prostate gland include proteolytic enzymes, acid phosphatase, and IgA. The final region is the bulbourethral gland which makes up 1-5% of the volume of semen through the production of mucoproteins (6). A healthy, fertile male typically produces over 15 million spermatozoa per milliliter and more than 39 million sperm per ejaculate (7).

The most common constituents used for semen detection in forensic casework are sperm cells, prostate-specific antigen (PSA), semenogelin (Sg) and acid phosphatase (AP). Semenogelin is produced in the seminal vesicle and plays a role in seminal coagulation (8). High levels of Sg are found in semen while lower levels are present in various tissues throughout the body. AP is present and detectable in several different body fluids such as saliva and vaginal secretions but is found in abundance in semen. A common colorimetric test used to detect semen relies on AP to catalyze the hydrolysis of a phosphate ester into a chemical that reacts with diazonium salt (8). This causes the formation of a colored precipitate, and the speed at which the color change occurs is correlated to the AP level, thus can be indicative of the semen concentration of the sample. For example, a semen stain with a dilution of 1:20 might create a color change within 60 seconds while a dilution of 1:100 may create a color change after 90 seconds (9). A delayed reaction can also be due to the presence of AP from non-semen source.

Prostate-specific antigen is another component of seminal fluid that is found in high concentrations even though it can also be found in fecal material and sweat in low concentrations (10). Detection of PSA is another commonly utilized in laboratories to attempt to identify a seminal stain. A typical way to detect PSA uses a chromatographic immunoassay that works through the placement of a stain on a porous membrane in the presence of a monoclonal PSA antibody (11). The monoclonal PSA antibody is linked to a dye and in the presence of PSA, an antigen-antibody complex forms (12). The complex

migrates up the membrane and interacts with the polyclonal PSA antibody that is located at the test region and creates an antibody-antigen-antibody sandwich. The visual representation of this sandwich is a colored line that is formed on the PSA test card (12).

Since Sg, PSA and AP are all components of semen that can be detected in other body fluids, their detection is not proof of semen. The only confirmatory method for the detection of semen is a positive microscopic examination for the visual identification of sperm cells, or spermatozoa. Sperm cells are identified by the morphological features of an intact sperm cell or a sperm head. An intact sperm cell is comprised of three mains parts: the head, midpiece and tail. The head of the sperm cell contains the nucleus and includes a cap-like structure on the anterior portion called the acrosome. The midpiece hosts the mitochondrial spiral which provides energy to the sperm for mobility, and the tail propels the sperm toward the egg. To aid with the visualization of cells such as sperm and epithelial cells, staining techniques are utilized to create contrast [Figure 1]. Two common staining techniques are the Kernechtrot picroindigocarmine stain (KPIC), also referred to as Christmas tree stain, and the hematoxylin and eosin stain (H&E) (13). When utilizing the Christmas tree stain the acrosomal cap of the sperm will appear a lighter color pink/red than the posterior head, while the midpiece and tail, if present, appear yellowish green (14). This color change occurs as the nuclei in the spermatozoa and epithelial cells are exposed to nuclear fast red. The color change of the midpiece and tail of the sperm as well as the cytoplasm of epithelial cells occur because of their exposure to picroindigocarmine. The

H&E staining works similarly to the Christmas tree staining, however, the nuclei are stained purple due to their exposure to hematoxylin (15). The midpiece and tails of the sperm cells as well as the epithelial cell cytoplasm appear pink from the interaction with eosin Y (16). Using these staining techniques aids with microscopic visualization of a single sperm cell, though slides containing few sperm cells or significant amounts of debris can be challenging to evaluate.

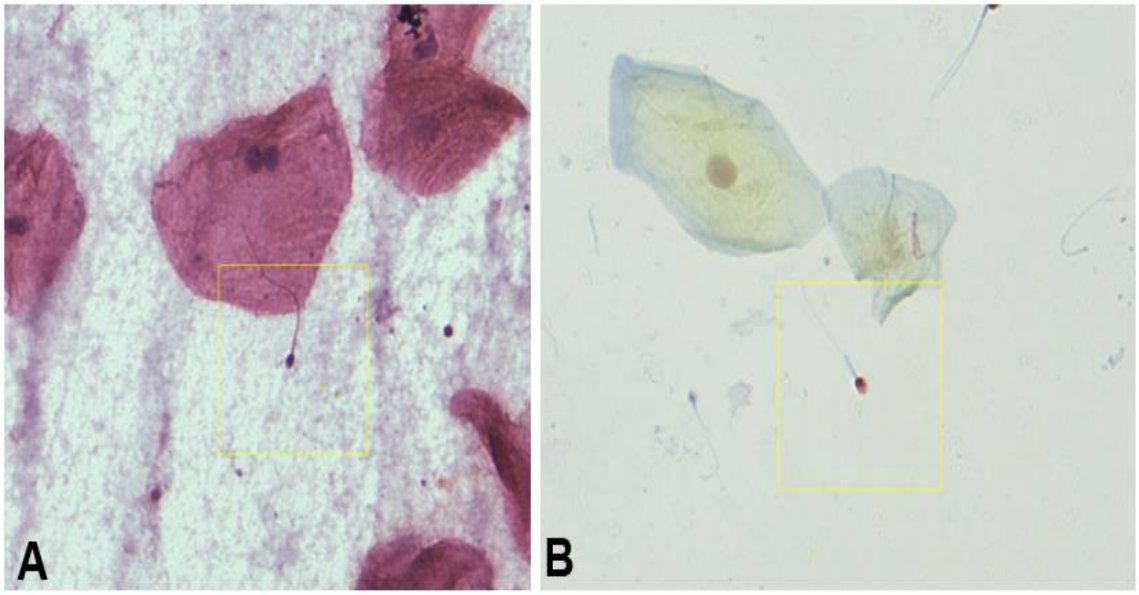

Figure 1. Microscopic view of sperm cells and epithelial cells stained with KPIC and H&E under 100X. (A) Sperm cell and epithelial cell stain with H&E. (B) Sperm cell and epithelial cell stained with KPIC.

Microscopic visualizations of sperm cells are a time-consuming, but important part of sexual assault evidence examination that are frequently performed prior to DNA extraction. Depending on the condition of the evidence and experience level of the analyst,

a microscopic examination can take anywhere from a few minutes to an hour. The task can also be physically taxing for an analyst since it requires looking into a microscope lens or computer monitor for long periods of time.

1.2 Automated Microscopes

Automated microscopy can be traced back to the 1960s. It was first created by researchers at Cambridge-based company called Metals Research. The group developed an analyzer for microscope images by using a television camera as an input device (17). Automated microscopes were created to improve the level of data processing as well as reduce inconsistencies that can arise from manual analysis when using a traditional microscope. Automated microscopes allow slides to be scanned and processed by a computer while reducing the work required by an analyst. Over the years, automated microscopes have undergone significant development that has led to them to become more sophisticated and versatile. A major change occurred in the 1990s with the implementation of image analysis with a computer-based Quantimet 520 which allowed image analysis to be fully software-based (17).

Automated microscopes have been used in a wide variety of applications such as scientific research, medicine, and art conservation. They can be found in research in fields such as microbiology, pathology, and pharmacology. Automated microscopes are used to examine tissue samples and even the effects of drugs on cells (18). Conservators utilize

automated microscopes when conducting pigment analysis and detailed cleanings or restoration of paintings that require high degree of precision (19).

1.3 Metafer and Artificial Intelligence Technology

Artificial intelligence (AI) has been a rapidly growing field over the last couple of years and its application can be found in many different areas. AI technology works by training a machine to mimic the functions of a human mind as the machine is taught problem-solving skills and can learn different methods (20). AI is mostly trained through an algorithm that contains information regarding a field or niche skills the machine will have to carry out. AI can be found almost everywhere from music, medical diagnosis, manufacturing robots, and self-driving cars (21).

In 1986, MetaSystems (Altlussheim, Germany) began manufacturing and designing computerized automated microscopic imaging products. The MetaSystems Metafer system includes a compound microscope with a motorized stage, high-resolution digital camera, and a software platform that uses specialized algorithms (classifiers) to recognize and group objects of interest. These classifiers or machine learning algorithms work by utilizing two different approaches to AI learning. The first method is a rule-based system that sets predefined rules to specify how the system can respond to different inputs such as images and sizes. The rules are created by humans and are used by the algorithms as a guide for analyzing images. The second method is through a machine learning algorithm. This algorithm allows the system to learn from data that is inputted and improve its

performance over time. Metafer conducts this learning through a supervised manual review that provides labeled training data. This combination allows for an effective AI system for microscopic examination.

The Metafer software specifically uses artificial intelligence and machine deep learning to analyze prepared microscope slides and identify sperm cell candidates. Metafer's imaging system uses magnification and contrasting methods to locate a variety of target objects. Its ability to locate objects under magnification allows for it to apply to many fields including forensic science, pathology, toxicology, and hematology. MetaSystems uses an internal interface for the organization of cases and data collection. This allows users to access the slide images even after the images have been captured on the microscope. Each classifier is trained based on parameters that are set by the user and can be improved as more data is interpreted and learned by the AI. This training allows for the improvement of the accuracy as the AI is provided with more data in its algorithm.

1.4 Purpose of Research

The microscopic visualization of sperm cells can be a tedious task for forensic scientists, but it is an important skill for analysts to have, especially as part of sexual assault evidence examination. Using an automated system that is equipped with AI to locate and identify sperm cells may help to alleviate the challenges of using a manual microscope by reducing the time it takes to analyze each slide manually and allowing for more efficient quality control during the review process.

This research evaluates the MetaSystems microscope and Metafer software for the forensic identification of sperm cells and compares its efficiency and accuracy to that of traditional microscopy. Samples containing semen as well as epithelial cells and a variety of contaminants were included to simulate stains found on evidentiary items (20).

2. MATERIALS AND METHODS

2.1 Preparing Semen Dilutions

Semen and saliva samples were donated by anonymous volunteers to the Boston University Biomedical Forensic Sciences Laboratory in accordance with a protocol approved by the Institutional Review Board.

Part I of the research assessed how well the Metafer system could detect sperm cells in different dilutions of semen. Three dilutions were prepared using semen with a sperm count in the normal range [Table 1]. Saliva was used as a dilution medium so that epithelial cells would be present in the sample and represent epithelial cells that can be found in mixed biological fluid stains. Additionally, a control sample absent of sperm cells was prepared. To simulate stains that are found outside, a sample was prepared by adding soil to a dilution of 1:100 semen and water.

Table 1. Part I samples created for blind analysis.

Contaminant Type	Semen Dilution	Contents
Epithelial Cells	1:100	2 µL semen + 198 µL saliva
Epithelial Cells	1:250	2 µL semen + 498 µL saliva
Epithelial Cells	1:500	2 µL semen + 998 µL saliva
Soil	1:100	2 µL semen + 198 µL water

Once the samples were created, 3 µL were deposited onto 12-well multi-spot microscope slides (Shandon, Pittsburgh, PA) [Figure 2] and the author was blinded to the locations of each sample type. The slides were heat fixed and stained prior to analysis.

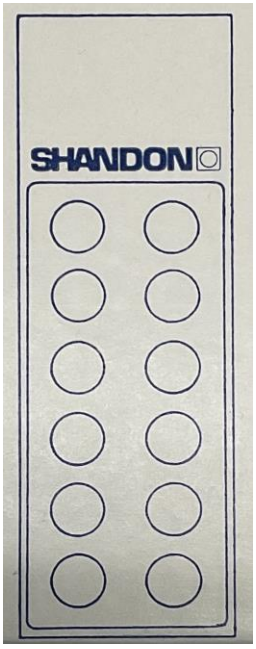

Figure 2. Shandon 12-well slides used for all samples.

Part II of the research evaluated sperm cell identification in the presence of biological contaminants and utilized semen samples that were determined to have low sperm counts. Six sample types were prepared: semen in water, semen mixed with saliva, and semen mixed with two bacteria strains, mold, or yeast [Table 2]. Contaminants were purchased from a commercial vendor (Carolina Biological Supply, Burlington, NC) and included *Staphylococcus aureus*, *Lactobacillus acidophilus*, *Candida*, and *Aspergillus niger*.

The contaminants were removed from their tubes using a scoopula that was sterilized with 70% ethanol and passed twice through a flame of a Bunsen burner. Once the scoopula cooled, it was used to collect a small amount of contaminant that was then

deposited into the labeled microcentrifuge tubes. This cleaning step was conducted for each scoopula before and after it was used. After the contaminants were transferred, 30 µL of a 1:100 semen dilution was added to each tube. Each sample was briefly vortexed then 3 µL of each sample type was deposited onto the 12-well slides. There were 5 repetitions of each sample type for a total of 30 samples. Slides were heat fixed and stained prior to analysis.

Table 2. Part II samples created with semen and contaminants.

Contaminant Type	Semen Dilution	Contents
None	1:100	2 µL semen + 198 µL water
Epithelial Cells	1:100	2 µL semen + 198 µL saliva
Staphylococcus aureus	1:100	30 µL 1:100 semen:water + *Staphylococcus aureus*
Lactobacillus acidophilus	1:100	30 µL 1:100 semen:water + *Lactobacillus acidophilus*
Candida albicans	1:100	30 µL 1:100 semen:water + *Candida albicans*
Aspergillus niger	1:100	30 µL 1:100 semen:water + *Aspergillus niger*

2.2 Staining

Heat fixing of the slides was conducted on a hot plate set at 100°C to firmly adhere the samples to the slide. The slides were heat fixed for approximately 1 minute or until the samples were visibility dry.

The heat fixed samples were first stained using one drop of nuclear fast red stain (XMAS Tree Stain A, SERI, Richmond, CA). The stain was left to absorb into the samples for 12 minutes, then the slides were rinsed with distilled water until the water ran clear. The slide was heated again for 20 seconds at 100°C to allow for the evaporation of any remaining water. Once the slide was dry, one drop of picroindigocarmine stain (XMAS Tree Stain B, SERI, Richmond, CA) was added to the samples. The stain was allowed to absorb for one minute before rinsing clear with a gentle stream of ethanol. The slides were allowed to air dry before sealing. Four small drops of Cytoseal mountain media (Cytoseal ™ 60, Richard-Allan Scientific, Kalamazoo, MI) were added to a coverslip (Fisherbrand ™ Microscope Cover Glass, Pittsburgh, PA) then the coverslip was placed over the slide. The cytoseal was allowed to spread out under the coverslip to ensure that the entire sample was covered.

In part III, a total of 10 samples - five samples using the 1:100 dilution of semen in water and five samples with the 1:100 dilution of semen in saliva - were stained with H&E. One drop of hematoxylin stain (Thermo Fisher Scientific Inc., Waltham) was added to each sample and allowed to absorb for 3 minutes. Once the stain was absorbed, samples were rinsed with a gentle stream of methanol until clear. The slides were placed on the hot plate for 10 seconds to allow the methanol to dry before applying the second stain. One drop of eosin Y stain (Thermo Fisher Scientific Inc., Waltham) was added to each sample, and allowed to absorb for 3 minutes. The slide was placed again on the hot plate for 10 seconds

to dry. Once the samples were dry, the slide was covered with a glass coverslip using one drop of Cytoseal ™.

2.3 Manual Sperm Counting and Scoring System

Each slide was manually analyzed using a binocular microscope (Nikon Eclipse E200, Nikon, Tokyo, Japan) under 400X magnification. The presence of one or more sperm cells was considered positive for the presence of semen and the absence of sperm cells in a sample well was recorded as a negative result. The slides were scored using a grading system to help with estimating the concentration of sperm cells [Table 3].

Slides were manually screened for sperm cells using a strip search method. This involved starting from the bottom left corner of a sample well and examining each field of view while moving the slide to the right. Once the right edge of the sample well was reached, the slide was adjusted upwards by one field of view, and scanning continued in the reverse direction. For the samples in Part I, only ten fields were searched if a sperm cell was identified. Samples were considered negative only after the entire well had been searched and no sperm cells were identified.

Table 3. Sperm scoring system.

Value	Observation
0	No sperm observed on slide
1+	Few sperm in some fields; difficult to locate
2+	At least one sperm in most fields
3+	Several sperm in most fields; easy to locate
4+	Many sperm in most fields

2.4 Operating Metafer

Up to eight slides were added to the MetaSystems microscope stage at once [Figure 3]. To begin scanning and capturing, the slides were named, then the search button was pressed. Manual focusing must be performed on the first slide to set the focal parameters that are used through the automated search. After the parameters were set, the slides were scanned using a 20X objective. The run was complete when the scanning ended, and images of each object appeared on the Metafer analysis review screen.

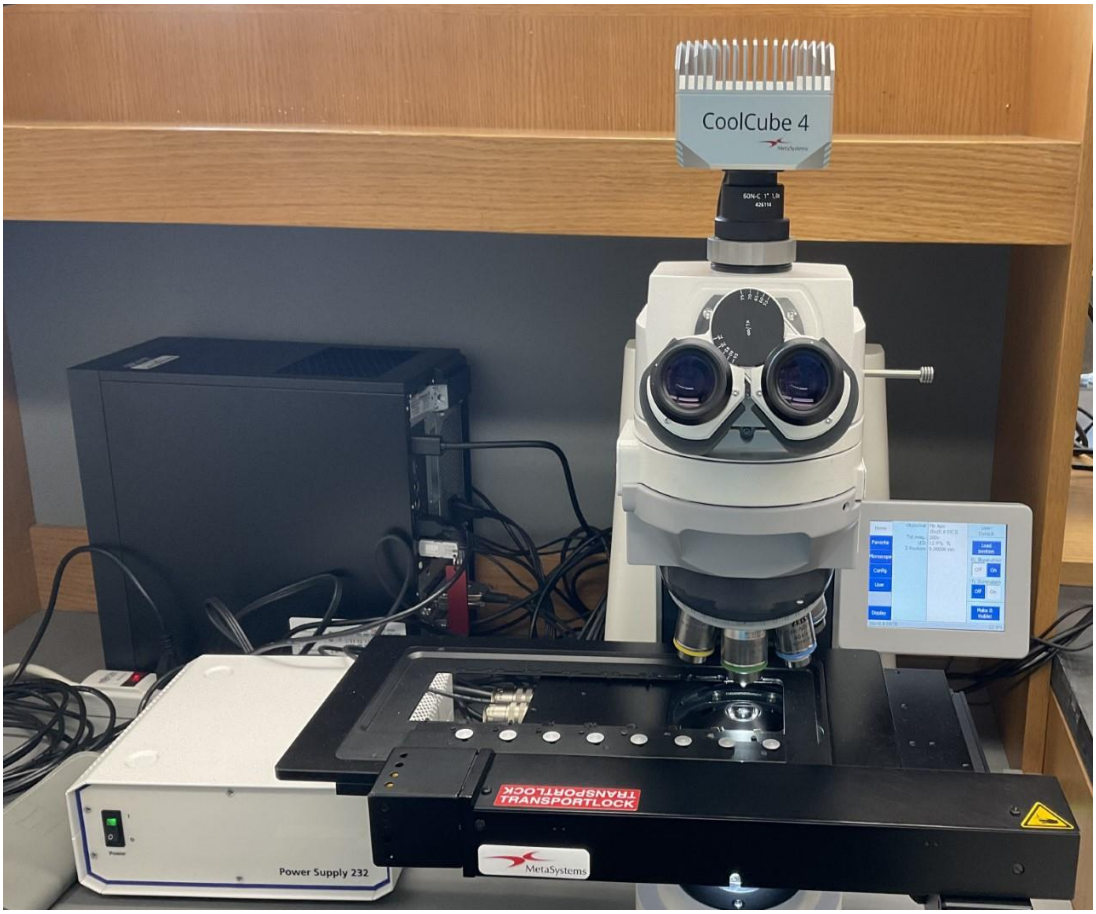

Figure 3. Metasystems automated microscope.

The slides were reviewed by selecting each tile on the Metafer review analysis screen. Each tile represented an object that was detected by Metafer, and they were sorted by the software into three categories: sperm candidates, unsure or background. As each tile was clicked, the microscope moved to the location or field of the object that was analyzed. This allowed the user to switch from stored gallery images to a live view.

Each tile displayed showed one sperm candidate that was highlighted with a green square. For example, if there were 400 tiles under sperm candidates then there were 400 individual sperm cell candidates detected by the software. Each tile contained four numbers in each of the four corners [Figure 4]. The top right number is the object detection score, or the score given by the software to reflect how likely the tile image contains an object of interest and the confidence level associated with it. The bottom right number is the probability of the object being a sperm cell as determined by Metafer. The more probable sperm candidates are displayed at the top of the screen and the probability decreases as the user scrolls down. The bottom left number represents the category in which the tile has been placed. The three categories are: 1 for background, 2 for unsure and 3 for sperm cell candidate. The probabilies assigned by the Metafer software are used to determine the category that the object/tile is placed into. Below the tiles of the images, bar graphs representing three different groups of objects identified by the software were displayed [Figure 5]. Finally, the number on the top left is the cell number of the tile image.

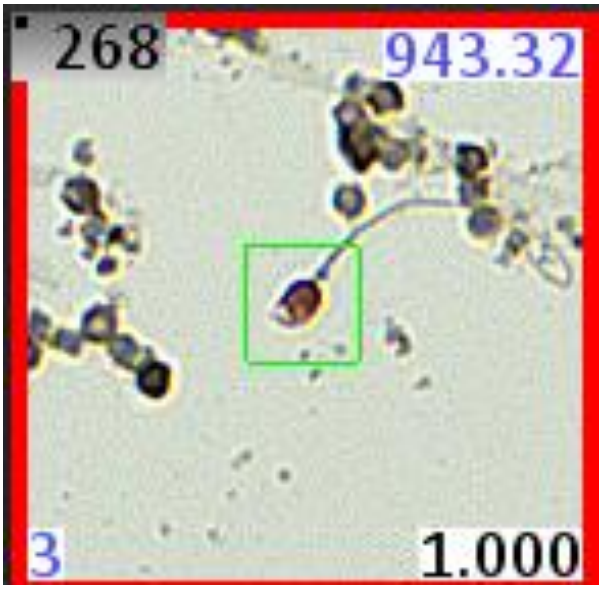

Figure 4. Metafer tile. Tile number = 268, object detection score = 943.32, probability = 1.000, tile category = 3 (sperm candidate)

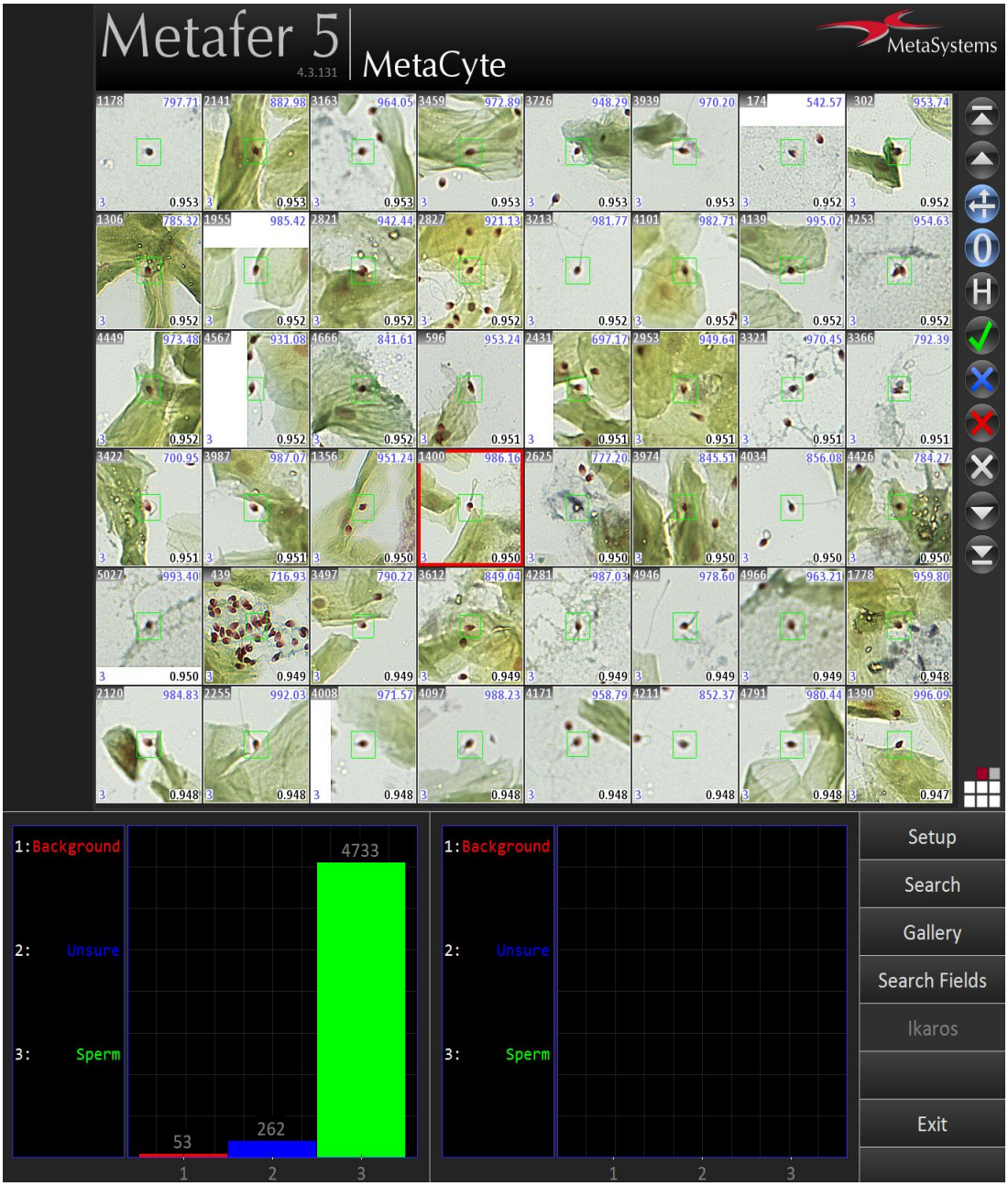

Figure 5. Metafer analysis screen. Visual representation of tiles with sperm image, probability, and location.

All objects identified by the software as possible sperm candidates appear as thumbnail images that include a quantitative value correlating to the relative strength of the classification. As a manual review of each tile is conducted, Metafer allows the user to modify the label it assigned to each object as a sperm, background, or unsure [Figure 6]. This enables a tally of the reviewed tiles and results of the manual inspection. Tile images are stored on Metafer and can also be examined using a live view feature which allows for further analysis and refocusing on objects of interest. Metafer also allows the addition of other groups of objects that may have been identified in the sample.

When manually evaluating the objects identified on the slides, the designated sperm cell candidates were reviewed beginning with those assigned the highest probabilities since these objects were identified by the software to be the closest to the morphology of a sperm cell.

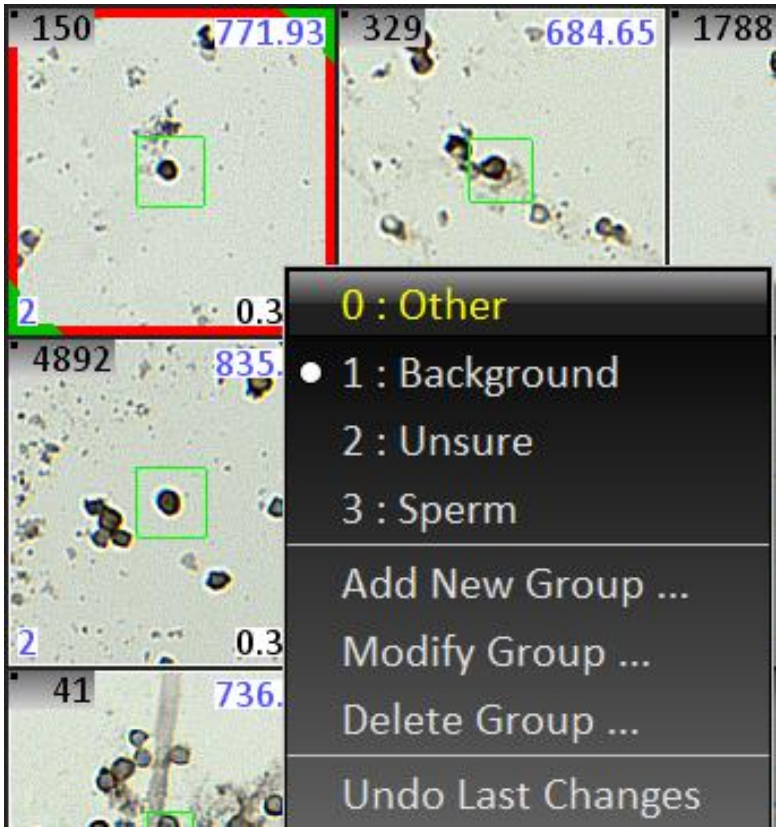

Figure 6. Sorting options for manual review. Manual change of tile from unsure (2) to background (1). Once the user makes a modification, the change will be reflected in the tile.

3. RESULTS AND DISCUSSION

3.1 Part I: Sperm Identification In Automated and Manual Search Samples Using Christmas Tree Staining

The MetaSystems microscope used in this research allowed up to 8 slides to be scanned and analyzed at a time though other models permit up to 800 stained slides to be loaded at a time. Each classifier is trained based on parameters that are set by the user and can be improved as more data is interpreted and learned by the AI. This training allows for improvement of accuracy as the AI incorporates data in its algorithm. The classifier utilized was trained using the Christmas tree stain and identifies cells by size and morphology. These settings allow for differentiation between epithelial and sperm cells [Figure 7].

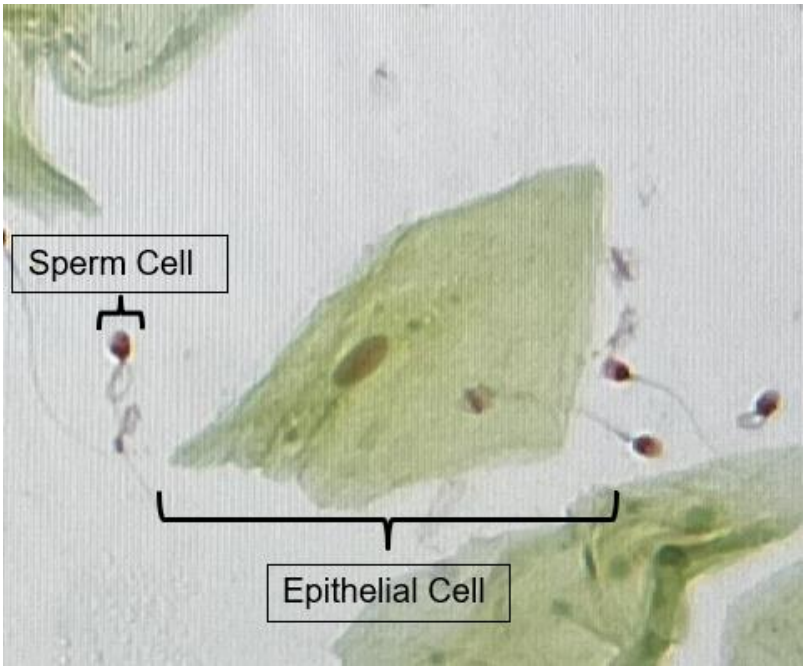

Figure 7. Microscopic view of the relative size of sperm cell vs. epithelial cell under 100X magnification.

Part I of this study compared the ability of the Metafer software to detect sperm cells mixed with soil and saliva compared to manual microscopy. This was done through a blind analysis whereby the author was unaware of the composition of each sample type to limit bias when manually analyzing the slides for sperm cells or confirming objects identified through AI as a sperm cell. Twenty samples were manually scanned and scored from 0 to 4+ using a Nikon microscope. After the manual analysis was conducted, the slides were scanned using the Metafer software. It is important to note that the manual review process of the slide on Metafer takes an additional 1-2 minutes to verify the sperm candidates detected. These extra 1-2 minutes are not reflected in the recorded time for the

Metafer search, which represents only how long it took the software to scan the images and perform the analysis that provided the list of sperm candidates. As familiarity with the software increased, the time it took for the manual review of each tile decreased.

The manual scores given to each sample were then compared to the sperm candidate amount assigned by Metafer. The line of best fit demonstrates that increases in the manual search score correlated overall with an increase in the number of Metafer sperm cell candidate results [Figure 8].

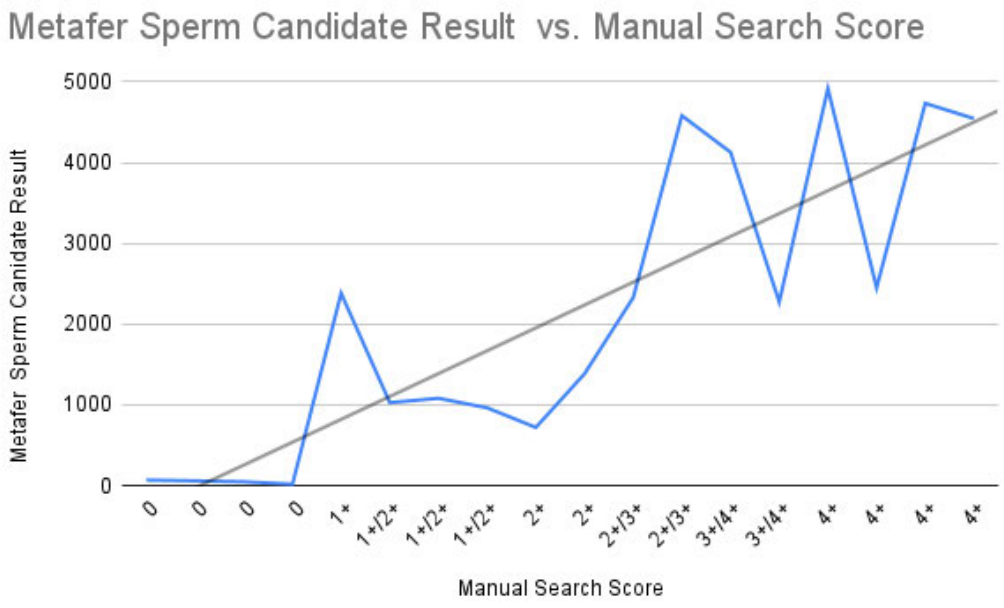

Figure 8. Metafer sperm cell candidate results vs. manual sperm search score.

The software rapidly identified all sperm cell candidates and displayed them as thumbnail images organized according to the strength of the match criteria as determined

by the classifier. When necessary, a tile image was selected and further analyzed by the user through the live view feature that allowed for zooming in on the objects. Further, Metafer successfully identified sperm cells amid epithelial cells and soil. Notably, this analysis included samples in which no sperm cells were observed during the manual search, demonstrating Metafer's ability to identify sperm cells that were missed during traditional microscopy. However, the software also identified objects as sperm candidates that were ruled out when the images were subsequently subjected to a manual review. This issue was more prevalent with the samples that contained soil. This may be attributed to the fact that the classifier was derived from training samples containing only sperm cells in water with no added debris (M. Corbaci, personal communication, June 6th, 2022). For that reason, the misidentification of soil particles as sperm cell candidates was not surprising since the software had not previously encountered this contaminant. Additionally, there were instances of sperm cells being located during the manual search that were not identified by Metafer during the automated analysis process. Most of the sperm cells missed by Metafer were out of focus during the scanning process. During the Metafer review process, the live view function allows the analyst to adjust the focus of sperm cells that were missed initially by the software.

Overall, the Metafer sperm cell candidate results correlated with the manual search score that was assigned. Samples given a score of 1+ or 2+ by the author generally had fewer sperm cell candidate results compared to the 4+ samples which had higher numbers

of sperm cell candidates. Along with the score, the time it took for the manual analysis was also recorded [Table 4]. The timer began from the moment the slide was loaded onto the stage until a score was assigned. For slides on which sperm cells were observed, only 10 fields were analyzed. For the slides where sperm cells were not detected, the entire slide was analyzed before assigning a score of 0.

Table 4. Manual search time vs. Metafer search time.

	Manual search time (min:sec)	Metafer search time (min:sec)
	1:21	0:30
	1:52	0:21
	2:40	0:28
	2:47	0:19
	2:54	0:23
	2:55	0:23
	3:06	0:22
	3:07	0:20
	3:13	0:23
	3:29	0:21
	3:31	0:29
	3:53	0:14
	3:56	0:20
	4:01	0:30
	6:03	0:14
	6:34	0:29
	6:53	0:15
	7:35	0:14
	8:14	0:15
	9:48	0:15
Average	**4:23**	**0:21**

The manual search time ranged from 1 minute and 21 seconds to approximately 10 minutes. Conversely, the Metafer search time fell between 14 seconds and 30 seconds. This difference in time to locate sperm cells and estimate sperm concentration is one of the primary benefits of an automated microscope. During the scanning process, slides were loaded on the stage, configured, then scanned. The entire process for all twenty samples was under 10 minutes and the scanning of individual samples took on average of less than 30 seconds. This ability to load the slides and then walk away as they are scanned allows analysts to perform other duties in the lab and return for the review process. Since the tile images of the sperm cell candidates are displayed in order of probability as determined by the classifier, the efficiency when reviewing slides once the scanning is completed is also increased. Additionally, the Metafer data allows an analyst to reasonably estimate the number of sperm cells present in the sample. Such information is important for downstream DNA analysis as it may inform the way DNA extraction and typing is performed.

Faster manual search times were usually correlated with samples that had higher sperm cell counts, while longer times corresponded with samples comprised of lower

sperm cell concentrations. In contrast, there was little variation in how long it took for the analysis to be conducted using the Metafer system.

3.2 Part II: Sperm Identification by Metafer Amid Varying Contaminants

Part II of this research focused on the ability of Metafer to identify sperm cells amid other cell types that may contaminate forensic samples. In additional to epithelial cells, the cellular contaminants selected were:

- Mold - *Aspergillus niger*
- Yeast - *Candida albicans*
- Bacteria - *Staphylococcus aureus*
- Bacteria - *Lactobacillus acidophilus*

A. niger also known as “black mold” is a common mold that is found in soil, water, the air, and decomposing matter. It also grows on fruits and vegetables such as peanuts, onions, and grapes. The *A. niger* spores appeared small, dark, and circular. The size and shape of the mold was similar to that of tailless sperm cells, which posed a challenge for Metafer during the analysis [Figure 9]. The similar morphology led to mold spores being identified by Metafer as sperm cell candidates.

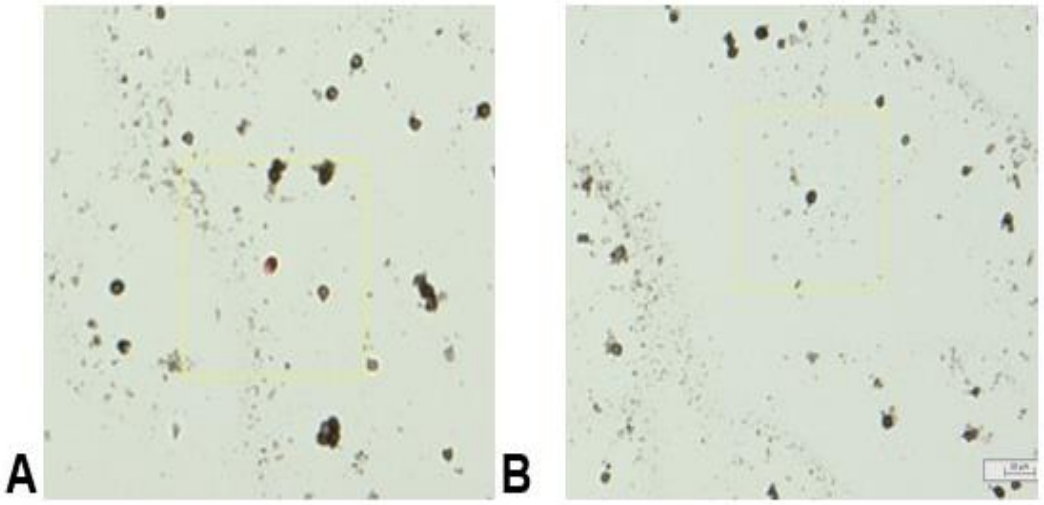

Figure 9. Objects classified by Metafer. (A) Metafer identification of tailless sperm cell amidst *A. niger*. (B) Metafer misidentification of *A. niger* as sperm cell candidate.

The second contaminant selected was *C. albicans* [Figure 10]. *C. albicans* is a fungus that can be found in the normal human microbiome (22). It commonly lives in the mouth, skin, and intestines. This naturally occurring yeast is responsible for vulvovaginal candidiasis, a yeast infection of the vaginal cavity and tissues of the vagina that affects approximately 75% of women in their lifetime and can increase during pregnancy (23). In sexual assault casework, it is not unusual for *C. albicans* to appear in samples originating from an individual's vagina. *C. albicans* was abundant in the background of the slides and increased the difficulty of identifying sperm cells due to the density of the cells. The similarity in color and dense layer reduced the contrast between sperm cells and *C. albicans*.

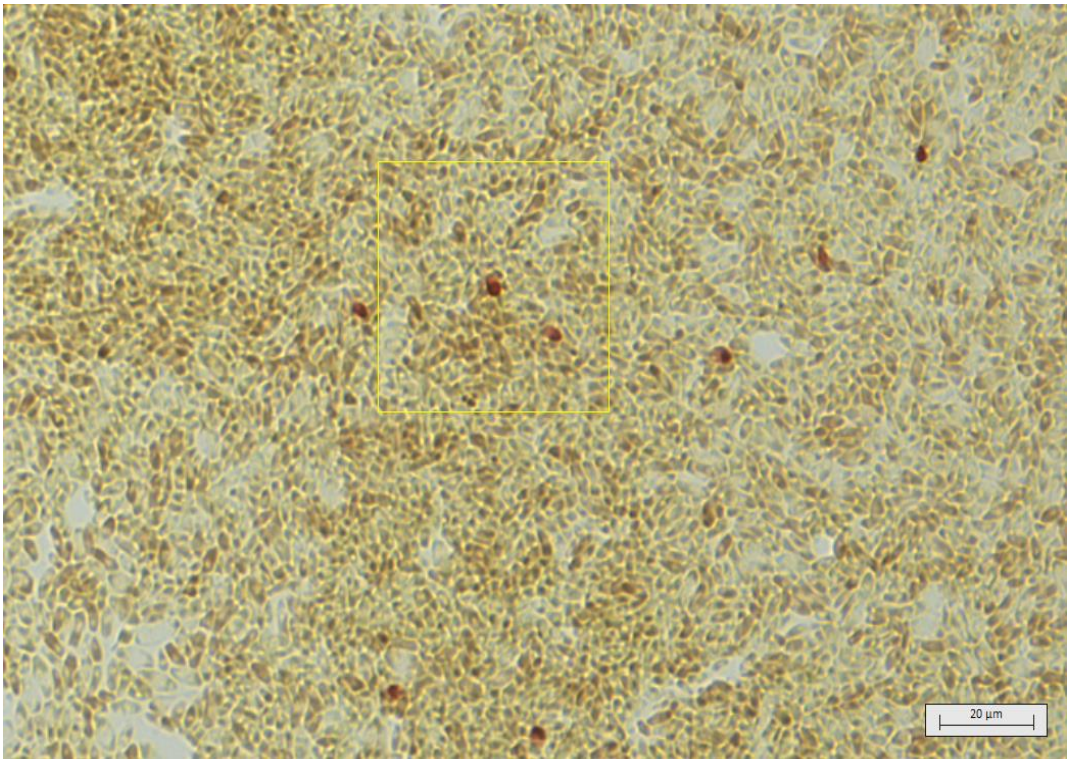

Figure 10. Metafer image of sperm cell in *Candida albicans* sample.

The final two contaminants included were *S. aureus* and *L. acidophilus. S. aureus* is a bacterium that is found in normal human flora on the skin or nasal area of healthy individuals (24) [Figure 11]. It is usually not harmful but can sometimes cause clinical

infections such as pneumonia and bacteremia (25). Metafer performed well with identifying sperm cells in the midst of *S. aureus*.

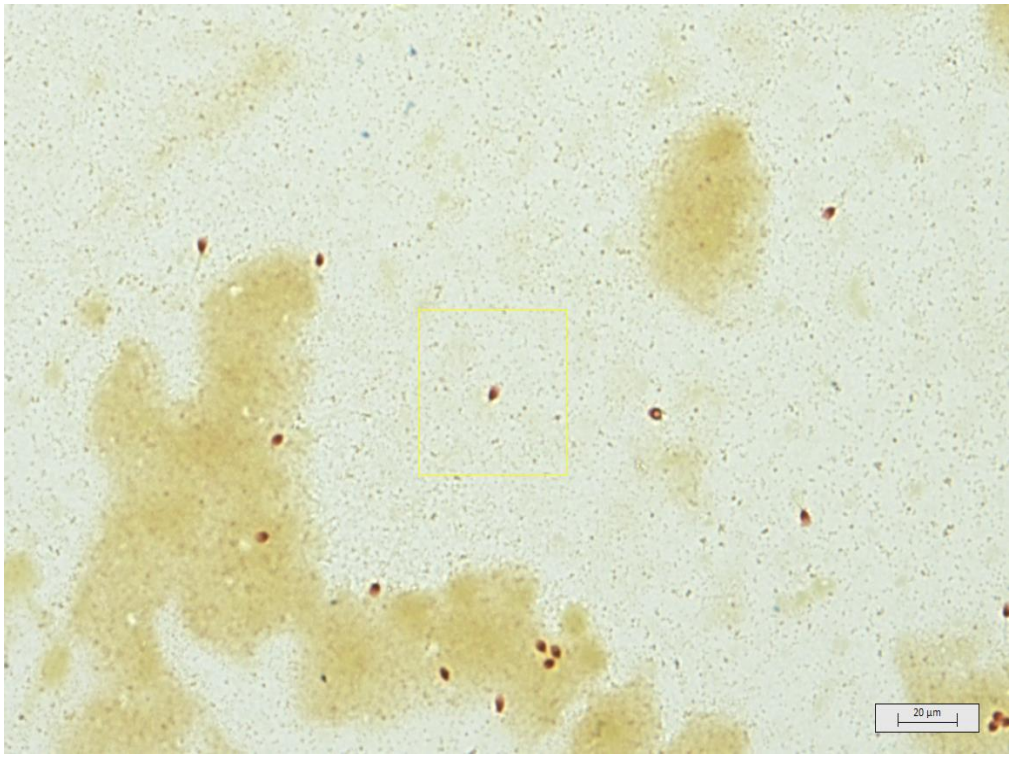

Figure 11. Metafer image of sperm cell in *Staphylococcus aureus* sample.

Lastly, *L. acidophilus* is a lactic acid bacterium that is commonly found in the oral cavity, gastrointestinal tract, and vaginal cavity [Figure 12] (26). *L. acidophilus* is considered a good bacterium as it has beneficial characteristics that aid gastrointestinal and general health. These benefits are part of the reason it is advertised commercially as a dietary supplement (27). The presence of *L. acidophilus* increased the density of objects in sample; however, Metafer was able to identify the sperm cells present.

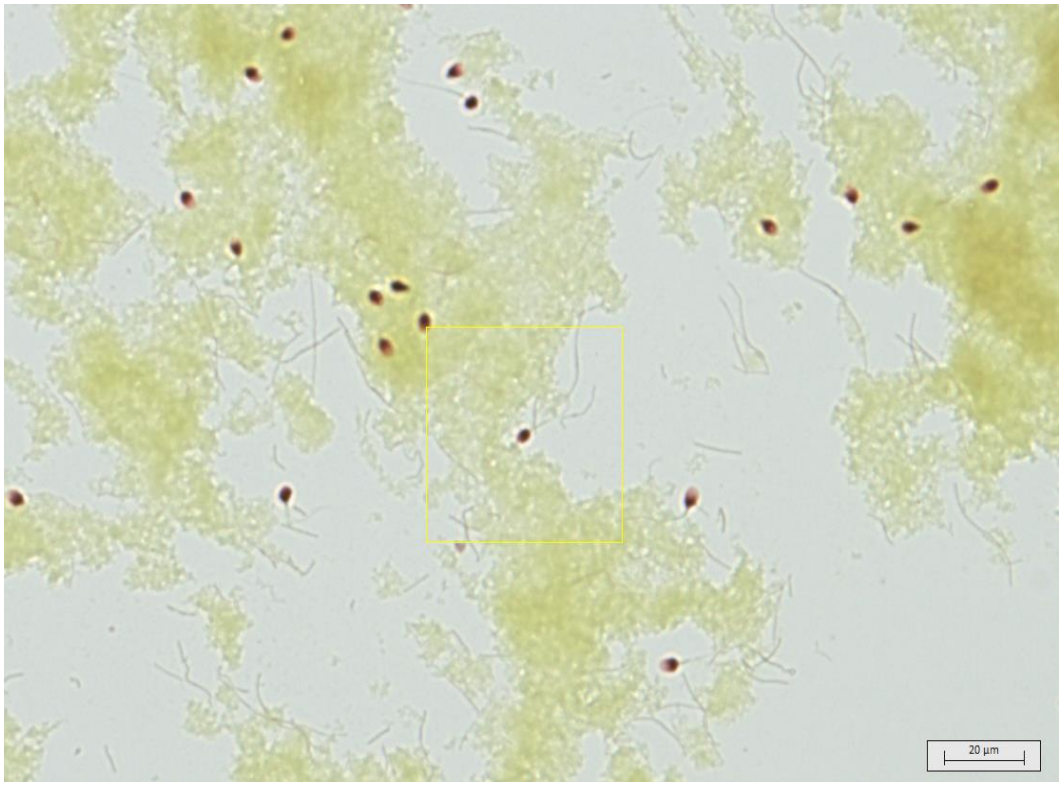

Figure 12. Metafer image of sperm cell in *Lactobacillus acidophilus* sample.

Each contaminant was combined with a low sperm count semen sample that were stained with Christmas tree and analyzed both manually and with the Metafer system. Samples with low concentrations of sperm were utilized to increase the difficulty of detecting sperm cells. Additionally, half of the samples were mixed with saliva to introduce epithelial cells. The results demonstrate that Metafer was able to identify sperm cells in samples with just a few sperm cells, and it outperformed manual search methods in terms of number of sperm cells detected [Tables 5-10].

Table 5. 1 in 100 dilution sperm:water stained with Christmas tree. Number of sperm cells observed with manual microscopy vs. number of sperm cell candidates detected by Metafer.

1:100 Sperm:Water	
# of sperm observed manually	# of sperm candidates detected by Metafer
1	1
7	3
2	2
2	6
1	2

Table 6. 1 in 100 dilution sperm:saliva stained with Christmas tree. Number of sperm cells observed with manual microscopy vs. number of sperm cell candidates detected by Metafer.

1:100 Sperm:Saliva	
# of sperm observed manually	# of sperm candidates detected by Metafer
0	0
0	5
1	0
0	5
0	2

Table 7. 1 in 100 dilution sperm:saliva with *Aspergillus niger*. Number of sperm cells observed with manual microscopy vs. number of sperm cell candidates detected by Metafer.

Aspergillus niger	
# of sperm observed manually	# of sperm candidates detected by Metafer
0	0
0	0
0	0
1	0
0	0

Table 8. 1 in 100 dilution sperm:saliva with *Candida albicans*. Number of sperm cells observed with manual microscopy vs. number of sperm cell candidates detected by Metafer.

Candida albicans	
# of sperm observed manually	# of sperm candidates detected by Metafer
0	0
3	0
0	0
0	0
0	0

Table 9. 1 in 100 dilution sperm:saliva with *Staphylococcus aureus*. Number of sperm cells observed with manual microscopy vs. number of sperm cell candidates detected by Metafer.

Staphylococcus aureus	
# of sperm observed manually	# of sperm candidates detected by Metafer
0	3
1	5
0	0
4	7
2	3

Table 10. 1 in 100 dilution sperm:saliva with *Lactobacillus acidophilus*. Number of sperm cells observed with manual microscopy vs. number of sperm cell candidates detected by Metafer.

Lactobacillus acidophilus	
# of sperm observed manually	# of sperm candidates detected by Metafer
1	1
2	2
0	0
2	3
3	3

In some instances, Metafer failed to identify sperm cells that were observed during the manual search. This issue was common when the parts of the slide were out of focus or in areas where the depth of field varied [Figure 13]. This issue occurred in at least one sample for each of the different contaminants. In these instances, the sperm cell was detected, but classified by the software as Unsure (category 2) because of the low probability value associated with it.

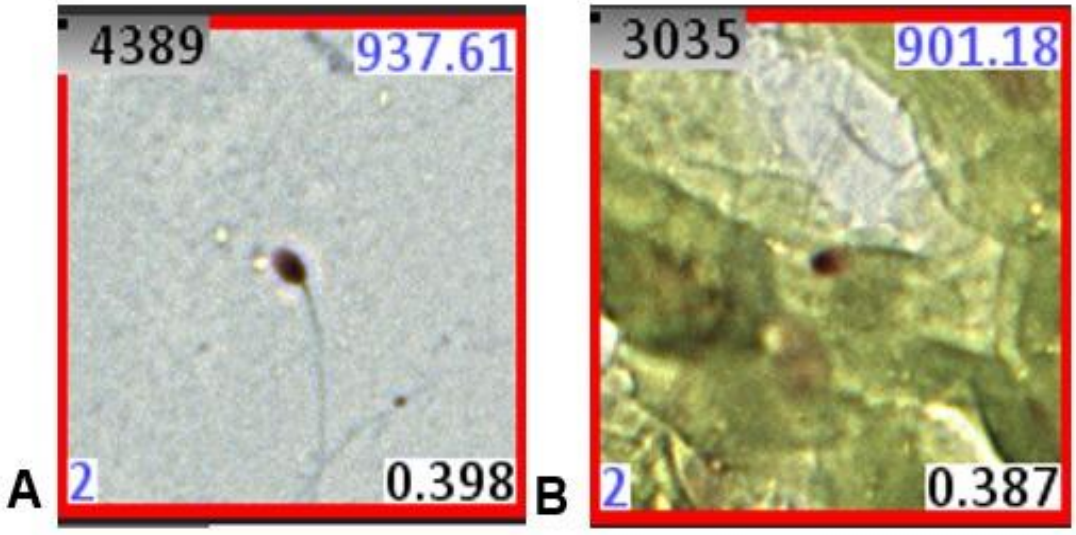

Figure 13. Sperm cells images affected by focal parameters. (A) Sperm cell out of focus. (B) Sperm cell under epithelial cell causing a difference in depth of focus. The software assigned these to the Unsure category.

Metafer was able to identify sperm cells with varied morphologies including intact sperm cells and tailless sperm heads. Additionally, sperm were detected with morphology anomalies such as thickened midpieces and coiled tails [Figure 14] (28).

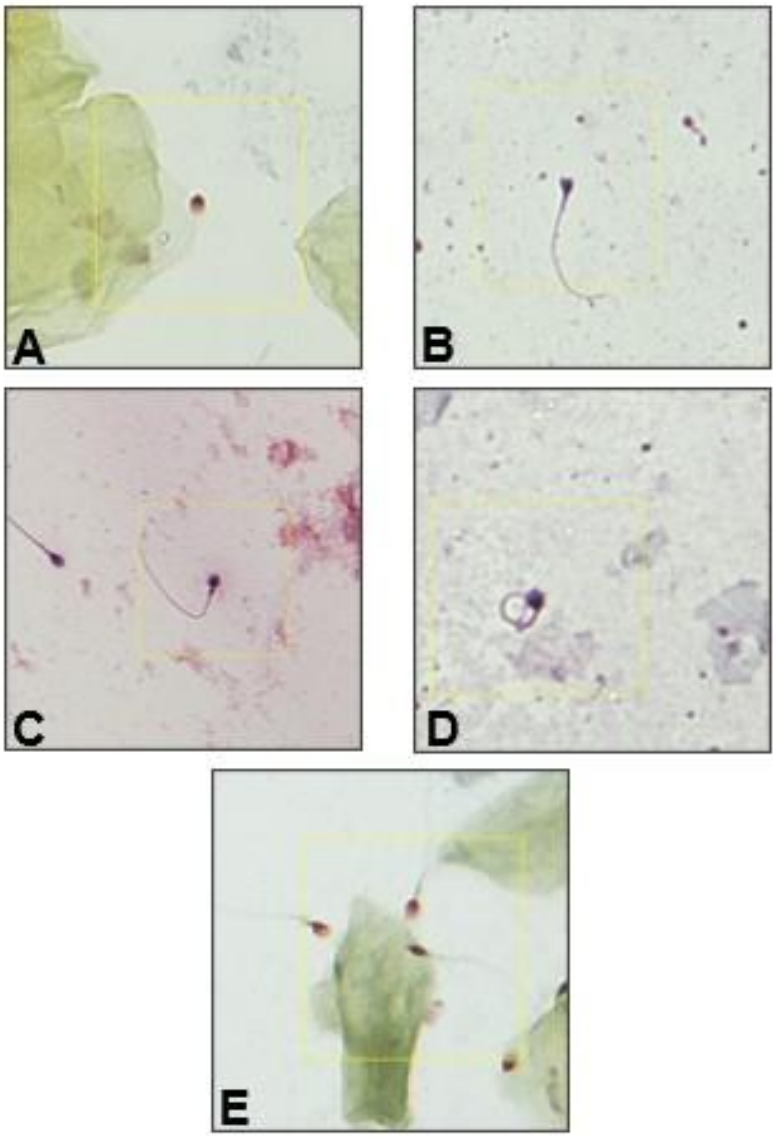

Figure 14. Examples of different sperm cell morphologies identified by Metafer. (A) Identification of a tailless sperm cell. (B) Identification of a duplicate sperm cell with two heads. (C) Identification of a sperm cell with a large swollen midpiece. (D) Identification of a sperm cell with a coiled tail. (E) Identification of a sperm cell with a thin narrow head.

3.3 Part III: Sperm Identification In Automated and Manual Search Samples Using H&E Staining

Part III of this study analyzed Metafer's ability to scan and identify sperm cells that were stained using H&E. Metafer software was trained using the Christmas tree staining technique, however, since the criteria for identifying sperm cells included size and morphology of the cells, it was hypothesized that the classifier might be able to identify the sperm cells despite using another staining technique.

A manual analysis was conducted on 10 samples to identify the number of sperm cells that were present. These samples type included neat semen and semen mixed with epithelial cells found in saliva. The amount of time it took to conduct a manual examination of each sample was recorded. The duration of analysis and number of sperm cells observed in each sample were compared to results using Metafer [Tables 11-14]. Again, there were instances of sperm cells not being identified by Metafer that were located during the manual search due to the sperm cells being outside of the targeted focal depth. Examples of this were observed in both conditions tested.

Table 11. 1 in 100 dilution sperm:water stained with H&E. Number of sperm cells observed with manual microscopy vs. number of sperm cell candidates detected by Metafer.

H&E 1:100 Sperm:Water	
# of sperm observed manually	# of sperm candidates detected by Metafer
4	13
13	14
6	16
7	0
6	20

Table 12. 1 in 100 dilution sperm:water stained with H&E. Manual search time vs. Metafer search time.

H&E 1:100 Sperm:Water	
Manual search time (min:sec)	Metafer search time (min:sec)
8:12	0:19
7:29	0:18
6:24	0:19
6:32	1:10
7:10	0:17

Table 13. 1 in 100 dilution sperm:saliva stained with H&E. Number of sperm cells observed with manual microscopy vs. number of sperm cell candidates detected by Metafer.

H&E 1:100 Sperm:Saliva	
# of sperm observed manually	# of sperm candidates detected by Metafer
2	2
5	7
7	5
6	5
2	5

Table 14. 1 in 100 dilution sperm:saliva stained with H&E. Manual search time vs. Metafer search time.

H&E 1:100 Sperm:Saliva	
Manual search time (min:sec)	Metafer search time (min:sec)
9:00	0:18
5:05	0:19
4:33	0:18
8:01	0:18
5:02	0:20

Metafer successfully identified sperm cells stained with H&E and performed similarly to the samples prepared with the Christmas tree stains. These results indicate that Metafer was able to accurately identify sperm cells that were different in color from those included in the training set. It took Metafer an average of 27 seconds to scan and analyze the samples that were stained with H&E. The duration of time it took Metafer to scan and analyze the slides was comparable to that during the Christmas tree stain analysis. Similarly, analyst manual search time did not differ greatly between the two stain types. However, the time required to examine slides for sperm through manual microscopy was significantly greater than using Metafer.

3.4 Areas for Improvements

Metafer was successful in quantifying the sperm candidates in the samples. However, some samples containing *A. niger* and soil resulted in misclassifications due to the similarities in the size and morphology of these contaminants with sperm cells. Including these and similar contaminants in future classifier training sets may provide a method for circumventing these types of misclassifications.

Improvements concerning the depth and focus that the camera can capture would also be beneficial. With the semen samples contaminated with soil, at times the soil particles were layered on top of each other creating more than one depth of focus. This proved to be a challenge for Metafer since the focus parameters are set at the start of the scan using only one sample. These same parameters are then used for all eight slides and

does not take into consideration the different sample types that may be on the slides and the varying depths. There is an advantage to not having to adjust the focus for each slide or sample well as this increases the speed at which that the microscope can scan the slide, however, it may compromise the accuracy of the results. With slides of varying depths due to the sample type, it was not uncommon for the microscope to misclassify or fail to detect sperm cells that were not captured in sharp focus. To combat this issue, out-of-focus slides were identified during the manual review and subsequently visualized using the live view feature. The live view allowed the slide to be viewed in a more traditional manner whereby the focus was adjusted as needed. This option could also be useful in casework during the peer review process. Another method taken to fix this issue was by individually re-scanning a slide that was out of focus. This allowed the focus parameters to be set specifically for the depth of that slide, however, it increased the amount of hands-on time required by the user.

3.5 The Future of Automated Microscopes in Forensics

There are multiple benefits of incorporating an automated microscope into a forensic laboratory's workflow. Currently, forensic biologists at the Vermont Forensic Laboratory are using the MetaSystems microscope and have reported it to be useful in casework (29). Microscopy coupled with AI allows for an increase in the productivity of a small laboratory as the hands-off nature of the automated scanning and analysis provide more time for an analyst to conduct other tasks. Decisions regarding downstream testing

can be made with confidence in a shorter period of time. Likewise, the efficient verification process and electronic image capturing may strengthen a lab's quality control measures. These images can be stored on Metafer or can be exported to other platforms connected with Metafer.

Automated microscopes can have a place in forensics not only in sperm cell identification but also for other sample types such as trace evidence. The ability of being able to utilize multiple different types of classifiers or algorithms on the same microscope can increase the microscopes application to multiple displines within a single laboratory. Automated microscopes are equipped with high-resolution cameras and imaging software that can capture detailed images of objects. This can be helpful with the examination of evidence such as fibers, hairs, and other small objects (30).

Implementing this technology in forensics will allow for an increase in the speed and efficiency of laborious tasks while improving the accuracy of examining sexual assault slides. Due to the time it takes to manually analyze slides it has become more common for forensic laboratories to forgo microscopic analysis altogether and submit evidence directly for DNA analysis. Although this approach eliminates the time required for microscopic analysis, it increases the cost of DNA processing as well as affects prosecutorial systems. In some cases, the detection of sperm in sexual assault cases can alter the types of charges brought to a defendants. Microscopic analysis is part of the traditional analytical scheme of forensic semen testing because it screens out false positive samples that can arise from

conducting an AP spot test or other presumptive testing methods. Additionally, the microscopic examination of slides allows an analyst to estimate the number of sperm cells and other types of cells that are present in a sample. The quantity of sperm cells that are in a sample can affect the extraction method that is selected for DNA analysis as well as the amount of sample that is used. Likewise, microscopy allows an analyst to estimate the amount of male sperm cell DNA that may be present in the sample relative to the concentration of female DNA from epithelial cells. The current method for separating these fractions in a mixture is through a differential extraction. This type of extraction is a two-step process for separating sperm cell DNA and non-sperm cell DNA (blood cells, epithelium, etc.) in mixed stains by taking advantage of disulfide bonds in the sperm cell head, which necessitates different conditions in order for DNA to be released (31). Since this process can take up to 8 hours in manual and automated differential extraction methods, automated microscopy is beneficial as it is less time consuming and can result in more precise information about the sample type.

Furthermore, automated microscopes can be used to analyze chemical evidence, including the identification and quantification of drugs and explosives. High-resolution cameras can increase the detail observed by a forensic analyst. Overall, the use of automated microscopes in forensic laboratories permits analysts to carefully examine and document evidence while making informed decisions quickly and accurately.

However, it is important to acknowledge potential issues that may arise when using automated microscopes. The first challenge is the high cost of automated microscopes, which can be prohibitively expensive for many laboratories. To combat this obstacle researchers have begun to develop and validate a low-cost optical system that utilizes AI technology (32). Additionally, automated microscopes cannot eliminate the potential for human error when operating the microscope. Analysts still need to conduct manual reviews, which require a level of skill and knowledge to ensure that the microscope is used effectively. If an analyst is not properly trained or does not follow the correct procedures, it may lead to errors or inconsistencies in the results. Lastly, automated microscopes are not infallible and there is potential for false positive or negative results due to different factors, such as background noise or other artifacts that the software algorithms may be unfamiliar with. This was an issue faced in this project, specifically with soil samples that appeared to confuse the software.

4. CONCLUSION

Metafer was able to identify sperm cells present in both high and low concentrations while also recognizing various sperm cell morphologies. The software was successful in identifying sperm cells that were treated with either Christmas tree or H&E stains. Additionally, the Metafer system was successful in sperm cell identification amid different cell types and contaminants, which often cause difficulties during a manual search. Metafer

also produced accurate and reproducable results when identifying sperm cells in a range of conditions. The main challenge faced was that some objects appeared out of focus when scanning multiple slides in a single run. This was due to the fact the focal parameters could only be set once. Sperm cells that were out of focus could still be identified during the manual review but were placed by the software in the unsure or background categories. Live refocusing during the manual review process was necessary in these samples to confirm the presence of a sperm cell. In conclusion, the use of an automated microscope and AI software such as the Metasystems microscope can increase the throughput of sexual assault samples in a forensic laboratory with improved accuracy compared to traditional microscopy techniques.

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