Development of an automated system to detect spermatozoa on laboratory slides to increase productivity in the analysis of sexual assault cases

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ABSTRACT

The analysis of a sexual assault case by the forensic laboratory is a multi-step procedure. One step in this process is often a lengthy microscopic examination of slides produced from samples collected from the victim's body, such as vaginal smears, and also from other crime scene evidence to determine the presence or absence of spermatozoa. This manual search for sperm can take considerable time depending upon the nature of the slide. Since the identification of sperm and the number available is a good indicator of the potential success of a subsequent STR analysis, there is a need to develop a procedure that allows swift analysis of these slides. The "faster, more robust, and less labor-intensive identification of sperm in the analysis of DNA evidence" can assist the forensic scientist in determining the appropriate use of resources and of crime scene samples. A method for quick and accurate screening of slides for sperm could decrease the turnaround time for sexual assault cases, more accurately determine which cases would be suitable for autosomal versus Y STR analysis, and re-direct staff to assist in other aspects of the biological analysis of the case. Such processing and analysis could give the analyst valuable information to assess samples, saving time and money that could be directed to other analyses.

The proposed project intended to explore the possibility of using existing hardware and modifying software to develop an automated sperm searching system. The system would allow the analyst to load multiple slides into the device for unattended analysis. Verification of the computer identified spermatozoa would be performed through the inspection of captured images or through a computer-driven directed review of the slide. The proposed system would consist of a microscope, a computer-driven stage that would accept multiple slides, a video system to import images into a computer, and software to drive the stage and to interpret images.

This study, at first, involved only one vendor; however, it soon included a second vendor with the hopes that two systems, used in comparison, would speed the process of developing one working system. The initial objective was to use the commonly used stain, referred to as Christmas Tree stain, that requires the microscope to use the normal bright field light system. This grant was expanded to include the use of fluorescence staining, such as DAPI or FITC, so each automated microscope was required to have the ability to switch to a fluorescent lighting and detection system.

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EXECUTIVE SUMMARY

An automated system to examine microscopic slides for spermatozoa would be an improvement over manual examinations for many reasons, including but not limited to, more efficient use of forensic analyst time, faster examination time per slide, faster turn-around time for a criminal case examination, and reduction in repetitive and ergonomic injuries to the employee. For these reasons, the development of an automated system was proposed for this grant. The casework slides were made from crime scene evidence collected by police or by sexual assault nurse examiners. Most often the slides submitted for examination for spermatozoa have been made from swabs used to collect evidence from the body orifices such as vaginal, oral and rectal areas.

The initial approach of the project was to use a bright field microscope produced by one company. The project soon expanded to compare two bright field microscopes produced by two different companies to determine which system worked better. A comparison of two systems was intended to increase the speed of the development of one working system. Also tested was the possibility of using fluorescent dyes or fluorescently tagged antibodies specific to human sperm cells for view with a fluorescent microscope. The goal of the fluorescence aspect was to provide a fast screen of potential sperm cells under fluorescence and with the ability to then switch to bright field in order to truly identify the sperm (i.e. a double stain approach). Both automated microscopy systems purchased included a microscope, a stage that held multiple slides, a computer system to process the images, and a screen to view the gallery of images collected. The systems were capable of scanning using bright field or fluorescence.

The automated bright field microscopes identify the spermatozoa by using a camera to capture the images of each object seen on the slide. The computer processes these images and judges by the size, shape, and color whether the object is a sperm cell or not. The slides are stained with a stain referred to as the Christmas Tree stain since it colors the sperm red and green (Oppitz, 1969). This stain, commonly used in the forensic community, is inexpensive and easy to use. The microscope developed by MetaSystems (Waltham, MA; Figure 1) was efficient from the day of its installation. It reliably found sperm cells including a single cell on a slide. It rarely missed identifying a sperm cell, which is extremely important since a sample routinely does not go forward for DNA testing unless a biological stain is identified. However, the slow speed of scanning the slide, often two hours per slide, was a significant problem since most sexual assault cases typically include three slides. Six hours to determine a result was far too long to wait during a normal work day.

The other automated microscopic system, developed by Loats Associates, Inc. (Westminster, MD; Figure 2), required more fine-tuning. This microscope and computer system had trouble focusing on the slides and, therefore, had trouble identifying sperm cells. The scans took far less time to run, often finishing in twenty minutes. However, the rapid run times for the Loats system did not produce quality results, as sperm were often not identified and focusing remained an issue. Both systems lacked the discriminatory power to distinguish sperm cells from debris or other cell types and reported numerous false positives.

The automated systems have software that allows the user to set the number of potential “sperm” object images collected for examiner review and verification. These selected images of presumed sperm are placed into a gallery. The gallery images are “snapshots” of the identified objects displayed as a “thumbnail” image in real-time as the microscope scans the slide. The

examiner can briefly review the images in the gallery as the microscope scans a slide or perform an in-depth review after the system has completed the analysis. Both systems identified too many false positive objects; items selected by the software as a possible sperm but upon review were either non-sperm cell debris or other unrelated microscopic material.

Both systems have large platform stages that allow multiple slides to be loaded for the automated run. The computer controls the stage, moving the slides under the objective for scanning; images are captured and displayed on a computer screen for viewing when the run is finished. The analyst is not required to do any further adjustments once the scope is started. After the run, the images can be studied on the screen or the computer can be directed to move the stage allowing cells to be viewed through the objective. The images on the screen can be marked as positive or negative for sperm and saved as a computer file. The slide can be removed, stored, and later returned to the stage for review. The file for a particular slide can then be opened and the stage moved to allow direct viewing of each identified cell. The gallery of photos and the exact coordinates can also be printed, if required, for inclusion in a forensic report.

For this project, using the microscope in the fluorescence mode had some potential advantages over bright field illumination. If the objects of interest were made to selectively fluoresce, a software program could more easily identify “bright” objects in a dark field versus the identification of visible objects that must be selected based on size, color, and shape parameters. The fluorescence option was explored via two fundamentally different approaches. One was based upon the use of selective dyes which fluoresce upon binding to DNA that would allow the visualization of the cellular DNA. The other approach was based upon the use of fluorescently-tagged antibodies specific for human sperm cells. Through a multi-step procedure, antibodies tagged with the fluorescent dye FITC were selectively bound to human sperm heads for the

purpose of sperm identification in the fluorescence mode of the microscope. Both approaches were attempted unsuccessfully and finally abandoned in favor of the Christmas Tree stain approach. This decision was based on a number of factors dual staining of the slides with Christmas Tree stain and a fluorescent stain could not be achieved, the use and disposal issues associated with hazardous dyes, and the interference of background fluorescence proved to limit the effectiveness of selective DNA staining dyes for this project. The antibody approach was costly, labor intensive, and had some background fluorescence issues which were major drawbacks in providing an inexpensive and rapid approach to screening slides.

During the comparison trials of the two automated systems, Loats went out of business and, therefore, improvements to the scanning system stopped. All work thereafter was focused on the microscope from MetaSystems. The computer programs, referred to as “classifiers”, were being developed and improved steadily. The system rarely missed a sperm cell and fewer and fewer false positives were being identified... However, the scanning runs were taking longer to finish each slide. The microscope was in use for casework, usually at times when the analyst was busy doing other activities, or the system was loaded with slides and run overnight. This allowed the analyst to perform other duties and return to review the gallery of images. Any slide that was determined by the microscope system to be negative for the presence of sperm cells was reviewed manually, with an occasional single sperm cell discovered. When the microscope identified a sperm cell, it was considered a large time saver for the analyst and a relief from the often ergonomically stressful manual slide scanning. The original microscope delivered by MetaSystems was eventually replaced after the focusing motor stopped functioning. The new scope, a Zeiss AXIO Imager Z2, was installed along with new software and a new LED light source which enabled the focusing to be more precise. With these new components, the

scanning time improved from two hours to an average time of twenty minutes. Now a routine case involving three slides can often be finished within one hour. This allows the analyst to forward results to the DNA section and the investigator in a more reasonable time period. The MetaSystems automated scope system has now been used in casework for several months and is proving to be very useful and a valuable asset in the forensic laboratory setting. The Serology analysts have been trained in its use and often employ it as the first choice for screening slides instead of manual microscopic searches.

MAIN BODY

I. Introduction

A. Statement of the Problem

The identification of spermatozoa is very important to the determination of the presence of seminal fluid in evidence of criminal sexual assault investigations. The search for sperm cells can take a significant amount of time in a forensic laboratory when performed on a traditional manual microscope. Decreasing the amount of time needed to identify these cells would increase the speed at which crucial DNA test results could be forwarded to the investigating police officers. The proposed study was to determine if an automated microscope could be linked to a computer that would search a slide for the spermatozoa without the constant monitoring of an analyst. An independent system would need to be fast, yet accurate, generating reliable results without fear of missing sperm cells. Added benefits of using an automated system would be reducing ergonomic stress injuries for the analysts by restricting the number of hours spent working with manual microscopes. In addition, since the automated scope would not need adjustments once running, the analyst could perform other duties in the laboratory during that time. A system developed would need to be simple and inexpensive so that it would be as useful as the traditional manual system and, therefore, easy to use by any analyst.

The long-term goal of this project was to develop a relatively inexpensive, simple, fast and automated screening procedure to identify spermatozoa on microscope slides. The specific objectives of this study were to 1) develop an automated sperm search system, 2) evaluate the

sperm search system, 3) determine the relationship between sperm number and DNA analysis success, and 4) disseminate the methods to the forensic community

B. Literature Citations and Review

Forensic DNA analysis is an important analytical tool which has been used to solve many serious crimes; however, the full potential of this technology has yet to be realized by the criminal justice system. The various groups that compose the criminal justice community need to work together to understand the limitations of the system and find ways to make incremental advances within the system to steadily increase the number of cases that can be processed. The forensic laboratory stands in the middle of that community, with law enforcement agencies that investigate and submit cases poised on one side and the judiciary arm on the other. Advances made within the forensic laboratory will impact the entire community. Today, many law enforcement agencies do not submit cases because of the backlog that exists within the forensic laboratory. Those cases submitted may not be analyzed for extended periods of time resulting in huge backlogs of unanalyzed biological material in many locations across the country.

Many avenues to streamline the analysis of biological materials are undergoing scrutiny and are subjects of research projects by the forensic community. Methods that reduce the hands-on time required to conduct a particular analysis with no reduction in the quality of the analysis will increase the efficiency and productivity of the laboratory. As most forensic laboratories are hiring limited, if any, additional staff and have limited space within their facilities, tools that speed the analysis of casework utilizing existing forensic scientists will see immediate results in

the processing of case backlogs. The tool we wished to develop has the potential to save valuable staff time and speed the analysis of cases reducing the backlog of unanalyzed cases.

The US Department of Justice, National Institute of Justice's June 2010 report entitled "2007 DNA Evidence and Offender Analysis Measurement: DNA Backlogs, Capacity and Funding", states that a survey conducted in 153 crime laboratories nationwide estimated there were more than 70,000 backlogged samples that await DNA testing (Hurst and Lothridge, 2010). The large majority of these cases require the examination of collected evidence for the presence of trace biological evidence in the form of seminal fluid. A procedure that could speed this analysis would reduce the turn-around-time for these cases and would give officers timely information to pursue possible leads. Officers who receive examination results after a considerable time period must overcome the factor of time which drastically reduces the success of criminal investigations. We intended to develop an automated sperm search system that will reduce the time it takes to perform an examination of those cases in which seminal fluid may provide useful information to identify a possible suspect.

Forensic laboratories will often employ one or more methods to identify seminal fluid. Seminal fluid contains a number of components that may be examined by the forensic scientist. The enzyme acid phosphatase is found in high levels in seminal fluid and, although not specific for seminal fluid, can aid in the detection of seminal fluid stains. Likewise, riboflavin, a component of seminal fluid, fluoresces under appropriate alternate light source wavelengths and can also assist in the detection of seminal fluid stains. Neither one of these tests, however, is conclusive for seminal fluid. Another examination detects the presence of P30 or prostatic antigen (PSA). Although methods designed to test for PSA may appear to have some cross reactivity with

materials of non-seminal fluid origin, these tests are generally considered conclusive for the detection of seminal fluid. In addition to PSA, other protein markers, such as semenogelin, are being used for identification of seminal fluid. Independent Forensics (Hillside, IL) sells the RSID™-Semen kit that utilizes antibodies specific for the human semenogelin antigen; it is advertised not to cross-react with other body fluids.

The microscopic identification of spermatozoa is the classical approach to the positive identification of seminal fluid. Using this approach, an analyst stains the microscope slide and then scans the slide under high power magnification (typically 100X to 400X) looking for sperm cells. Several different stain combinations are available including fuchsin/methylene blue, hematoxlylin/eosin, gentian violet/methylene blue and Christmas Tree stain (indigo carmine and nuclear fast red) (Suzuki and Oya, 1985). Our laboratory currently uses the Christmas tree stain (Seri, Richmond, CA). This stain was found effective and easiest to read in a comparison with alkaline fuchsin and hematoxlylin/eosin (Allery et al., 2000). With this stain, the indigo carmine stains the cell cytoplasm green and nuclear fast red stains the nuclei red. Thus, sperm cells appear as small red ovoids with a cap of clear to green, sometimes with tails, while epithelial cells appear large and green with a large red round nucleus (Figure 3).

Of the conclusive methods used to identify a stain as being of seminal fluid origin, performing the analysis for human semenogelin antigen or PSA are typically the fastest, although more expensive approaches. The techniques used to identify either of these two proteins may only take a few minutes of hands-on time to prepare. The microscopic method may yield results relatively quickly if numerous sperm cells are present, i.e. the observation of sperm after reviewing a few microscope fields would typically end the analysis of the slide. Those slides on

which sperm cells are rare or absent take approximately fifteen minutes or longer per slide to view. Sexual assault cases often contain multiple slides that must be analyzed, especially in cases for which limited details about the event are available. Thus, an hour or more can be spent on the thorough examination of these slides, a task which is both tedious and physically demanding.

DNA analysis of a sexual assault case typically requires sperm cells to be successful. Although there are some reports of a successful autosomal DNA profile from a vasectomized male's seminal fluid or a fluid without any visible sperm (presumably through white cells in the seminal fluid) (for example, Sibille et al., 2002), successful profiling is typically based upon finding sperm cells upon a microscopic examination of the sample. Since the tests for semen specific proteins, e.g. PSA and semenogelin, reveal the presence of a protein in the seminal fluid matrix, it does not correlate to the number of spermatozoa present and hence, is not a good indicator of the amount of sperm present in a sample. In our laboratory, we have both obtained DNA profiles from cases for which no PSA was detected and have failed to obtain DNA profiles on PSA-positive specimens. Our results in this area are not unlike those obtained in other laboratories. Through the careful examination of microscope slides, predictions concerning the possible success of DNA profiling can be made. An estimate of the amount of sample necessary to go forward with DNA testing can be determined and evidence relating to sexual contact may be given to law enforcement officials.

The implementation of a sperm search system in the forensic laboratory would automate a process that ties an analyst to a microscope for extended periods of time, and would greatly increase the efficiency and productivity of the biology section. We envisioned a computer

controlled microscope system with software designed to identify spermatozoa. The system would have a carousel to hold a number slides; each slide would be scanned through the microscope and interpreted by the software system. Those locations where a sperm cell was identified would be mapped and imaged. After a completely unattended scan of the slides, the analyst would review the identifications made by the instrument, either by reviewing the captured digital images, or by reviewing the actual slide locations (i.e. the software-driven stage will "take" the analyst to view potential sperm found on the slide in order to view the "sperm" image live through the microscope or on the computer monitor).

C. Rationale for the Research

There are thousands of sexual assault cases that require examination. Many of these are in forensic laboratories within lockers, freezers, and cold rooms. Other cases are held by law enforcement officers who are hesitant to submit cases to an already burdened lab. This situation must be remedied. Backlogs need to be reduced and cases submitted to the laboratory. We are hopeful that this project will speed the analysis of sexual assault cases and play an important role in attaining these goals.

II. Methods

Slide Preparation

Cuttings taken from stain material were placed into microcentrifuge tubes, wetted with ~200 µl HEPES buffered saline (HBS), mixed, and incubated for 30 minutes at 4 ˚C. The solid materials were spun out with centrifugation. A portion of the resulting pellet was transferred to a glass microscope slide (Cat # 22-037-240, Fisher, Pittsburgh, PA) and allowed to dry prior to heat fixation.

Christmas Tree Staining

To the fixed slide, 1-2 drops of Nuclear Fast Red (Solution A, SERI, Richmond, CA) were added for 15 minutes before rinsing the slide gently with distilled water. Next, 1-2 drops of Picroindigo Carmine (Solution B, SERI) were added for fewer than 10 seconds before rinsing the slide gently with ethanol. The slide was then allowed to air dry.

Fluorescent Staining

Acridine Orange: To the fixed slide, 50 µl of acridine orange (Sigma) staining solution (100 µg/ml final concentration in distilled water) were added and allowed to sit for 20 minutes at room temperature before rinsing the slide gently with distilled water and fixing with ethanol. The slide was then allowed to air dry.

DAPI: To the fixed slide, 50µl of DAPI (Sigma, St. Louis, MO) staining solution (0.01 or 1 µg/ml final concentration in methanol or PBS) were added and allowed to sit for 20 minutes at room temperature before rinsing the slide gently with distilled water and fixing with ethanol. The slide was then allowed to air dry.

SYBR Green: To the fixed and Christmas Tree stained slide, 50 µl of SYBR Green (Molecular Probes, Eugene, OR) staining solution (0.1 µl/ml final concentration in TE buffer) were added and allowed to sit for 5 minutes at 4 ˚C before rinsing the slide gently with distilled water and drying at room temperature in the dark.

SPERM HY-LITER™: Slides were stained according to directions provided by the manufacturer (Independent Forensics, Hillside, IL). To the fixed slide, 2 drops of Fixative Solution were added and incubated at room temperature for 10 minutes before gently rinsing with 1X Wash Buffer. Approximately 75 µl of Sample Preparation Solution + DTT were added and incubated at room temperature for 30 minutes before gently rinsing with 1X Wash Buffer. Two drops of Blocking Solution were added and allowed to sit for 30 minutes at room temperature before gently rinsing with 1X Wash Buffer. Lastly, 2 drops of Sperm Head Staining Solution were added and allowed to sit for 30 minutes at room temperature before gently rinsing with 1X Wash Buffer. The slides were air dried and mounted using 1 drop of Mounting Media.

Automated Sperm Search Procedure

The following is the procedure used with the current automated microscope system and software. As procedures or hardware change, and software is updated, different steps may be involved to

operate the system. The procedure detailed here is provided to allow the reader to assess the steps required to perform the automated analysis of slides.

To operate the MetaSystems microscope, first turn on the system including the power box, microscope body, and the computer. Open the Metafer 4 file and load slides using the 20X objective. To turn on the light, select "Filters > Transmission Filter Wheel > White". To adjust the light intensity, select "Filters > Lamp Intensity > 100". Select "Setup" and "#1" next. Use the current sperm Classifier program available, (ex.TL-20_090910-V365), redefined search window, circle size, and cell count (ex. 2000). The entire smear can be viewed if required. Select "OK" – the slide information will be visible along the bottom of the screen and the slide stage locations will be highlighted in red. Select "Search" – slide the push-pull rod for camera path deflection that is on top of the microscope to the left. This rod directs the image to either the objectives or the camera. When pushed to the left, one can view the slide through the microscope's objective to focus. Alternatively, if the rod is moved to the right, the image of the slide appears on the computer screen. The examiner manually focuses by observing the image and moving the fine focus knob accordingly. The light is then adjusted. If the screen is red, turn the light down and focus if needed. Select "OK" and the microscope will move to the next slide. These focusing steps are repeated for each slide and the microscope will start scanning when ready. Cover the objectives with the black covers. After the run is completed, review each slide by double-clicking the label at the bottom of the screen. The gallery is displayed and the candidate images can be reviewed. The microscope can "drive" to each identified object for visual observation through the objectives if desired. Sperm cells identified in the gallery may be highlighted in green.

III. Results

A. Statement of Results

Phase One: Microscope Assessments

The progress of the research can be divided into two distinct time periods. The first is the longer during which two microscopes from independent companies were assessed. The second phase is marked by the physical improvements made to one of the microscopes that fundamentally changed the viability of the scope for forensic applications.

Two automated microscopes were purchased; one from Loats Associates, Inc. and the other from MetaSystems. Both microscope systems included a camera to capture the image, a computer with suitable software to assess the image and an electronically controlled moveable stage capable of holding multiple slides. The Loats system had an additional computer processing unit that controlled the microscope's stage and focusing movements. Both systems could be used in either bright field or fluorescence, though bright field was the primary use investigated.

Historically, at the Vermont Forensic Laboratory the manual sperm search process has used the Christmas Tree Staining method. This is a popular method used widely in the forensic field and utilizes the Nuclear Fast Red and Picroindigo Carmine stains which gives the spermatozoa a red and green appearance. This staining method was used by both microscope system manufacturers to set the search parameters of the automated microscopes. Stained slides containing sperm cells were given to the company representatives to allow them to develop software to locate sperm cells on a typical slide from a sexual assault case. Both companies had developed software

designed to search for particular cells in the clinical setting and were asked to take what they had previously learned and apply that to our particular need. These slides may contain epithelial cells, bacteria, yeast, cellular debris or other material in addition to sperm cells. The software had to be designed to identify sperm cells based upon a number of factors that included color, shape, and size with allowances for the challenges often observed with forensic specimens. The forensic laboratory may encounter specimens (sperm cells) that appear somewhat different than "text book" cells or may not stain exactly the same way between specimens. The software had to allow for these variances but still not identify too many false positive items. To accomplish this, microscope systems identical to the ones located at the Vermont Forensic Laboratory were located at the company research facilities. Once changes were made to the software, the improved product was provided to the Vermont Forensic Laboratory to test on site. The examiner attempted to keep the staining of the slides as consistent as possible and within typical variations since a bright red head portion was needed for the microscopes to identify the sperm cells.

Both automated systems were immediately capable of identifying spermatozoa upon installation; however, both had similar problems. The most important issue was attempting a constant focus on the cellular material in the smear no matter how thin or thick the non-cellular debris was. From the start it was observed that the microscopes needed a focal point on the slide other than the cellular material present. This led to the purchase of "ringed-slides". These are glass microscope slides with a single white circle of consistent size and location printed on the surface. The microscopes were able to focus on the white ring, giving them an initial coarse focus. The fine focus was still a problem, especially if the smear was overly thick and uneven, which is often seen in hospital-prepared smears made from direct application of an oral, vaginal, or rectal

swab to the slide surface. Alternatively, slides prepared in the laboratory are from swabs and cuttings that have been extracted. The extraction process removes the cellular debris and sperm cells (if present) and allows the examiner to make a more uniform smear. However, some of these smears often had so little cellular material that the microscopes had trouble focusing during the scan process.

The speed of the automated scanning runs was also determined to be a problem early on in the project. The Loats microscope could scan a slide quickly, occasionally finishing in one minute. However, the quality of the scan and the ability of the software to identify sperm and not "miss" one was a problem. The examiners could watch in real-time as the microscopes moved from field to field. The Loats microscope would often focus on a field that contained an obvious sperm cell, but it would move on to the next field without marking the object as an identified cell. Performance was inconsistent as the next field on the slide may have contained a sperm cell that was identified and marked. In addition, the Loats microscope, as well as the MetaSystems microscope, could take over two hours to scan a slide, and still obtain false negative results (i.e. not identifying obvious sperm cells present on the slide). Both microscopes would find some of the spermatozoa in the smear but not all. This was especially common if the spermatozoa were intact with the tail still attached to the head.

The automated microscopes would also identify numerous false positive, non-sperm cell materials. Some of the miscellaneous items identified as spermatozoa were objects that were of similar color, shape, and size which is understandable since many of these objects would be something a human operator would also stop and consider. However, many were oddly shaped and colored objects, cellular debris, and stain aggregates with no resemblance to spermatozoa.

This observation that the automatic microscopes could be "confused" by objects clearly outside the defined parameters was disturbing. In addition, a setting on the microscope from MetaSystems can be adjusted to stop the scan once a preset (by the analyst) number of identified cells has been reached. The microscope counts these objects it mistakes as sperm cells, causing the scan to quit before the entire smear is viewed. Since the microscope is misidentifying debris as sperm cells, the count can be composed entirely of items that are not sperm cells. If the setting for the count is increased, the microscope scans more of the smear but takes an even longer time to complete the run. This problem of misidentifying debris as spermatozoa was of crucial concern and had to be resolved in order to make the microscope system an effective tool.

This balance between scanning the slide quickly yet correctly without the identification of false positive items became the main issue for the two companies to overcome. In addition, the issue of properly focusing the specimen had to be addressed; if the cells on the slide were out of focus, it was impossible to identify the sperm cells. Although the systems would identify sperm cells, they lacked the accuracy of a human operator and could take considerably longer to scan a slide.

The two microscopes also experienced specific problems based on the differences between how they were designed and built. As mentioned previously, the Loats system has a separate CPU to control the movement of the focusing and stage. Oftentimes, the CPU failed to initialize properly and as a result, the microscope would not focus or the stage would not move when the run started. To remedy this, the entire system had to be shut down and restarted. In addition, much information had to be entered manually in order to start a run; this was tedious and involved multiple windows of information to complete. If the company had remained in

business, we would have sought an improved software package to allow the examiner to enter relevant information in an easier fashion.

In contrast, the MetaSystems microscope requires minimal data entry prior to starting a run. Needed information is restricted to a single screen and involves only entering the slide names. The stage can be lowered which allows the slides to be placed on the stage much more easily than on a typical manual microscope. However, the stage would sometimes fail to move back into the correct location underneath the objectives. This malfunction would cause the computer to send an alert that the stage was out of focus and multiple steps had to be taken to remedy this. As a workaround this feature was no longer used. Finally, the microscope automatically saves the gallery photos of the objects identified with the corresponding data in large files on the hard drive. As a result, the memory on the hard drive was expended and the data had to be saved on an external hard drive. Alternatively, unnecessary files could be deleted from the system to free up memory.

Over the next six months, both company representatives visited the VFL to make improvements to their systems. The task of building a successful system proved more difficult than first thought by either vendor. Both companies updated their programs with slightly modified parameters to force the microscopes to identify objects based on color, shape, and size with the goal of scanning more efficiently to lower the run times without missing spermatozoa. One resolution was to change the color detection levels so that sperm cells that were not stained bright red, as is often seen in casework slides, could still be identified. In addition, the MetaSystems program was modified to detect sperm cells when mixed in with or positioned on top of debris and epithelial cells. For instance, previously when the edge of the sperm cell

overlapped another cell or debris, the size parameter had a difficult time recognizing the spermatozoa. With the improved programming, this was no longer an issue.

The Loats system developed other problems related to the computer software. Results were combined for runs if a slide was rerun under the same name. Furthermore, it did not always distinguish between runs or assign sub-numbers to keep the data separate. In addition, the mechanical controls stopped responding to the CPU and the stage would fail to return to a certain location on the slide preventing the identified cells to be viewed by the examiner. Often the stage would move to the extreme right and freeze requiring the system to be shut down. The mechanical controller CPU became so unreliable that a physical block had to be installed on the stage to prevent it from rising so high that it would push the objective through the slide.

In an attempt to correct the focus, Loats developed the "Color Adjustment Steps" to be performed at the beginning of a run. The percentages of the color balance were recorded and reported back to the software analysts at the company. Unfortunately these steps did not help. The runs remained out of focus and failed to identify sperm cells even when the color was balanced as requested. Additionally, the microscope was still having trouble focusing on the cells if the smear lacked considerable cellular material. This was evidenced during the scan as obvious spermatozoa were overlooked whereas debris was identified. Finally, the gallery of photos was out of focus when the slide was, in fact, in focus, which made subsequent evaluation of a possible sperm cell difficult

Shortly afterwards, Loats went out of business and no further improvements to the Loats system were possible. There was some discussion about another company taking over the business and

continuing the efforts. However, this never materialized and all work stopped on the Loats system.

Work continued with the MetaSystems microscope and incremental improvements were made. The programming on the MetaSystems automated microscope was updated and this clearly improved the focusing and reliability of the sperm searches. The sperm heads and intact cells with attached tails were both identified in heavy debris. The runs were completed in one hour or less if spermatozoa were present. However, if the slide was negative the scans still took one to two hours and identified hundreds of false positive objects. The high number of false positives would take a long time for the examiner to review and were often oddly shaped and the wrong color and size. The printing function was attempted, but the prints had no color although important information such as cell coordinates would be printed. At this point it was decided that printing was not an important function to be pursued.

MetaSystems continued to review the slides and data files we provided and made software changes for our review. In reviewing our data files of slide analysis runs, the company recommended a physical change to our work place and suggested placing the microscope on anti-fatigue mats to reduce vibration. This improved image quality and searching success. As a combination of efforts improved results, we established a set of standard slides to run to ensure that the microscope was operating the same over time. We found that the slides need to be re-stained if saved for more than a few months. Staining fades with time and hence assessment of the slides in relationship to the performance of the instrument becomes problematic without re-staining. Following each run, information is recorded in a chart that includes the cells found

(both manually and automated), the speed of the runs and any relevant comments (Table 1). The corresponding slides can then be re-run at a later date to check the reliability of the microscope.

Our assessment of the progress of the project had to this point been focused on three parameters: the time it took the microscope to view the slide, the number of false positive results and the number of false negative slides returned from the analysis. If the microscope took too long to assess a slide the instrument would not be of value in reducing the time necessary to process sexual assault cases. If the microscope produced too many false positive images it would tax the examiner unnecessarily to review all the images. Finally, a microscope that missed sperm cells would be of limited value to the community. It was very simple to assess the progress based on these criteria - the system was not suitable for the community. The instrumental run time was too long, the examiner review of the false positives was too time-consuming, and too many sperm were not identified by the system (false negatives).

Improvements to the algorithm to reduce the false positives and lower the chance of missing a sperm cell could work in opposition to each other. One of the problems with the automated searching of sperm, in contrast to that of searching for cells in a clinical setting, is that the samples found in forensics may not be optimum specimens and may have been exposed to materials or environmental conditions that may affect the morphology or staining characteristics of the sperm. From our work and correspondence with others in the field, we have found that sperm morphology and the typical staining of sperm cells may be altered in many forensic samples. In addition, sperm cells may be overlapped with cellular debris, impacting the ability of the software to detect size and shape - important sorting criteria. A system designed with an algorithm with the “flexibility” to capture images representing a range of sperm morphology and

staining characteristics and perhaps "hidden" within other cells, will yield an increase in the number of items "identified" by the system. This conundrum of trying to design a system that would not miss a sperm without identifying too many false positive items was resolved when the company designed the software to sort the collected images. Images were rated and those with a "high probability" of being a sperm could be placed in the beginning of the gallery. An analyst could then quickly review the gallery and stop the review when the images become poor candidates. This review takes a matter of seconds and hence the issue of too many false positive images became moot and not a factor in our analysis. The software could encompass enough variance to accept sperm possessing a range of characteristics.

This change in the software, sorting images within the gallery, was important, but we still were concerned with the speed of the system and false negatives. Further rounds of data collection, software modification, and provision of slides for the company's review continued. Finally we decided that the software programs to identify the sperm, referred to as "classifiers" were satisfactory enough to allow us to use the system for casework analysis. We based this decision upon the fact that the system rarely missed a sperm cell. However, we felt the scan times could be improved. Even with this time "handicap" the microscope was implemented into casework and allowed to run either overnight or when the analyst was busy doing other activities. This permitted the analyst to perform other duties and return to review the gallery of images when time permitted. When the microscope system identified a sperm cell, it was considered a time saver for the analyst and provided relief from the often ergonomically stressful manual slide scanning process. If sperm cells were identified on a slide, the results were used and no further analysis was performed. However, if the microscope failed to identify sperm cells, the slides were viewed manually.

Phase Two: Scope Improvements

The MetaSystems microscope was implemented for casework and during our initial use of the system the microscope experienced focusing problems and produced error messages. The company was contacted and MetaSystems determined that many of their microscopes were having a problem with the focus motor. A new scope, a Zeiss AXIO Imager Z2, was installed at the VFL to replace the original. This system included new software and an LED light source to replace the halogen lamp and filters employed by the previous system. The new LED lighting system employs an LED illuminator for which individual LEDs for red, green and blue are turned on selectively before individual color images are taken. The black and white camera determines the color by the illumination detected. Previously the halogen lamp with white illumination and a tunable LCD filter was switched between red, green and blue so the filter determined the color. In this system the computer drives the LED illuminator instead of the LCD filter and relates the colors to illumination detected by the camera. The LED illuminator is considerably brighter than the halogen lamp and associated filters. Also in the previous system the color, texture, and contrast was dependent upon the condenser settings and the focus. When these were not exact, images were not well focused. The implementation of the LED illuminator removed the need for a condenser and made the imaging process more robust. With these changes to the microscope it was easier to calibrate and gave better and brighter images. Other changes were also made to what are known as “focus offsets”. This was the first time these offsets had been used and resulted in an improved image quality (better overall focus and more consistent contrast) and the slides could be analyzed more reliably.

These changes marked what we now call Phase Two of our project. A remarkable transformation took place with the instrument. The scanning time was reduced from two hours to an average time of twenty minutes. With the new microscope, a routine case involving three slides can often be finished within one hour. This allows the analyst to forward results to the DNA section and the investigator in a more reasonable time period. To document our findings, following each run information is recorded that includes the cells found (both manually and through the automated microscope), the speed of the runs and any relevant comments (Table 1). Approximately 376 slides have been run using the new scope with LED light source and only 3 false negative slides were detected during this period. In each case one sperm cell was found manually, but none were found by the MetaSystems microscope. These omissions are believed due to an excess of debris on the slide or poor staining quality of the sperm cells.

The MetaSystems automated microscope system has been used in casework for several months and is proving to be useful and a valuable asset in the forensic laboratory. The Serology analysts have been trained in its use and often employ it as the first choice for screening slides instead of manual microscopic searches. Slides may be placed in the slide holder and reviewed later. False positive images are quickly screened and slides are easily assessed based upon a fast review of the gallery of collected images. The images are sorted from high to low priority and assessments are done in seconds. Sperm locations on the slide can be quickly recorded and the instrument can “drive” to a location on the slide for direct viewing of the object recorded in the gallery. The MetaSystems microscope now provides useful information in a timely manner for the serologist and reduces the time necessary to read slides. As it is still possible for the instrument to miss a

sperm, a manual search is still done for those slides that are negative (i.e. no sperm found by the instrument).

Experimentation with Fluorescence

Using the microscope in the fluorescence mode has some advantages over bright field illumination. If the objects of interest can be made to selectively fluoresce, a software program could easily identify "bright" objects in a dark field versus the identification of visible objects that must be selected based on parameters such as size, color, and shape. In discussion with the microscope manufacturers, they believed that the time required for the analysis of a slide could be drastically reduced with a system that was based upon fluorescence. The fluorescence option was explored via two fundamentally different approaches. One was based upon the use of selective dyes that fluoresce upon binding to DNA (Clark, 1981). This would allow the visualization of the cellular DNA. Most slides contain epithelial and sperm cells and when these cells are stained with the fluorescent dye DAPI, a difference between the cells in noticed. Sperm cells have a relatively greater fluorescent intensity, although confined to a smaller area, than epithelial cells. This observation could be useful for the sorting of possible candidate cells through a software program. The other approach was based upon a method that uses fluorescently tagged antibodies specific for human sperm cells. Through a multi-step procedure, antibodies tagged with the fluorescent dye FITC are selectively bound to the human sperm heads allowing the identification of the sperm cells in the fluorescence mode of the microscope. This approach was combined with a selective DNA dye to stain the chromosomal material in the cells. This was commercially available as a kit and had recently been introduced to the forensic community.

Therefore, one of our goals was to examine whether fluorescently stained cells could be incorporated as a means to increase the speed of screening slides containing potential sperm cells. Our thoughts were that slides could initially be examined under fluorescence after dual staining with a nuclear-staining fluorescent dye and Christmas tree stain; putative sperm cells located quickly via fluorescence could then be positively identified after switching to bright field. Our reason for dual staining with both a fluorescent and visual dye was that upon examination of sperm identified via fluorescent methods, the morphological information obtained (via fluorescence) was deemed inadequate to properly identify the sperm, thus the need for a visual observation with Christmas tree stain. The Loats system was used in conjunction with the fluorescence setting and SYBR Green and Acridine Orange. Both the SYBR Green and Acridine Orange stained the epithelial cell cytoplasm and other debris in the smear as well as resulting in non-specific clumping of the florescent label. Furthermore, neither stain appeared to stain the sperm cells or the nucleus of the epithelial cells. The SYBR Green was then combined with the Christmas tree stain, but the mixture prevented the normal red and green staining of sperm cells. Since SYBR Green and Acridine Orange have the disadvantages of being both expensive and involving time-consuming procedures, it was decided that they were impractical for the purpose of an automated sperm search.

Using the automated microscope from Loats, numerous studies were performed to increase the success of sperm searching through use of fluorescent staining, both as standalone dyes and in combination with Christmas tree staining. Slides composed of sperm cells or epithelial cells were prepared from stains dried on cotton cloth (sperm) or cheek swabs (epithelial cells). 4', 6-Diamidinio-2-phenylindole (DAPI) was the first fluorescent dye that was tested. Working stain

solutions of DAPI were prepared in PBS or methanol at both 1:10 (1 µg/ml) and 1:100 (0.1 µg/ml) dilutions and applied to epithelial slides. The four staining conditions were similar; all epithelial cells were visualized with DAPI-stained nuclei. However, the slides utilized were not highly cellular and therefore, the experiment was repeated using slides heavily coated with cellular material. The slides stained with DAPI dissolved in PBS were superior to those stained with DAPI dissolved in methanol. The methanol slides had a high amount of fluorescence in the background. The DAPI dissolved in PBS at the 1:10 dilution was crisper than the 1:100 dilution and was chosen for future experiments.

Numerous experiments were performed in an attempt to combine Christmas Tree and DAPI staining. To determine a workable staining procedure, the Christmas Tree components (Nuclear Fast Red ["red"] and Picroindigo Carmine ["green"]) were applied to the slides along with DAPI in various orders (i.e. red – green alone, DAPI alone, red – green – DAPI, DAPI – red – green, red/DAPI – green, red/DAPI, red – DAPI). The Christmas Tree staining was not as sharp when DAPI was present (red – green vs. red – green – DAPI), but the sperm cells were easily identified. However, the DAPI staining on the red – green – DAPI slide was diminished to the point that nuclei and sperm were not discernible under fluorescence. Although the Christmas Tree staining was improved, the DAPI staining remained poor when DAPI was applied prior to the Christmas Tree stain or with the red component (i.e. DAPI – red – green and red/DAPI – green). When only the red component of the Christmas Tree stain was used in conjunction with the DAPI, the Christmas Tree staining was acceptable, but the DAPI staining was only moderately improved. The most promising condition was when the red component was applied prior to the DAPI.

Additional slides were stained with the red component and DAPI with variations in the concentration of the red (undiluted, 1:2. 1:4, 1:10 made in water) and length of time the red was applied to the slides (5vs. 15 minutes). Unfortunately, sperm on the slides stained with the diluted red component were not easily visible. However, when undiluted red was applied to the slides prior to DAPI (for either 5 or 15 minutes), the results were promising. There was bright DAPI staining of the epithelial nuclei and sperm, and the sperm were detectable under bright field based on staining with the red component. The red staining is a more subtle pink color, but sperm were easily identified when scanning for color on the slide. No further studies were performed along this line of investigation, but future studies could easily be performed to determine if the combination of DAPI with Christmas Tree staining increases the efficiency of automated sperm searching.

Fluorescent staining was further evaluated using the MetaSystems scope, employing the nuclear-dye stains DAPI and FITC. The DAPI stain was much brighter than FITC, but cellular materials incorporated the dye in addition to the sperm cells. This attempt to add a fluorescent step to the scanning procedure added additional hands-on time for the analyst and yet did not move us closer to our goal of double-staining the slides with both a fluorescent and visible stain. This was our last attempt to couple these procedures and afterwards we focused our attention exclusively on making the Christmas Tree stain a viable method for automated scanning.

Instrument Assessment

Improvements in the software were made through an iterative process. New software was provided and sperm cells that were not identified by the software were recorded. Those images

of non-identified spermatozoa and slides were sent to the company to make suitable software modifications to allow the software to "identify" those sperm cells in future versions. We continued this process but were not satisfied with the product based on three main criteria: scan time, false positives, and false negatives. These values could be tracked, but the same slide may give different values on different days and, hence, a relative assessment of the process rather than some numerical evaluation was conducted during most of Phase 1.

As noted above, a radical change in the project occurred after a new microscope with LED lighting was installed (see Phase 2). This new microscope and associated software produced faster scan results; results that would work for the forensic community. In addition, we had become proficient reviewing the gallery of images and with the sorting routine now included with the software; this process took only a few seconds to accomplish.

Two of our criteria for the scan speed and false positive rate had been solved. The scan speed from 2 minutes to 30 minutes should be appropriate for most forensic laboratories. The number of false positives is no longer an issue since most of those images are not "seen" by the examiner. This left one assessment: the false negative rate.

As a small laboratory we have a limited sample set for assessment. Nevertheless, during the months of the award during which the instrument was used, 376 slides were run and only 3 slides contained a sperm head that was missed by the system. These were sperm cells that did not stain appropriately or were positioned within debris.

To assess this instrument, one would have to ask if an unattended automated searching of a slide that takes from 2 – 30 minutes to complete, with a false negative rate of less than 1% is a useful piece of instrumentation to employ in the forensic laboratory.

Final Classifier Description

The classifier is the software employed by MetaSystems to conduct the cell selection process. Their description of the program is detailed below and is extracted from

This classifier detects sperms at 20x magnification. The classifier works with a TOFRA RGB LED light source. The classifier captures sperms assuming a white background for the Transmitted Light capturing mode. The classifier uses buffered capturing. During image acquisition, various image parameters are determined for each of the color channels – red, blue and green – to be used for further color transformation in order to give out the results in the correct colors at the end of the run. The cell selection algorithm used here is the one for single cells. This requires a definition of the minimum and maximum area of the cells to be captured in um2. It also requires that a concavity depth is defined for each single cell. The concavity depth is a criterion for determining single cells (the contour is convex with only a small concave area) and cell clusters (which usually have large concavities). Another parameter that is defined within this algorithm is the maximum aspect ratio of the cell i.e. the diameters of the long and short axis. This criterion is used to discriminate approximately round objects from more elongated ones. The cell selection algorithm within this classifier is set to select single cells with an object area between 5 and 22 um2, a maximum concavity depth of 0.750 and a maximum aspect ratio of 4.00. Non-elliptic cells and irregular cells are rejected by the classifier. The classifier is also set to process the image background and uses the operation SBHistoMax (Subtract Background

histogram maximum Position) where the gray level histogram is computed and its maximum is detected. The position of the Maximum is then subtracted from every image gray level. If the result is negative it is replaced by a 0. The SBHistomax is an aggressive background correction method resulting in cleaner image backgrounds in the final output. The SBHistomax is applied to all the three color channels in this case. No other image processing or cell processing operations are performed by this classifier. The classifier also records a quality criterion when acquiring images and at the end of a scanning session for a slide, sorts the images in order of quality within the gallery.

Predicting DNA Success

One of our goals was to determine if the number of sperm identified could predict DNA typing success. We did not actively pursue this. When we wrote this grant we thought that it may be useful to look at sperm count and corresponding DNA yields. This still may be a useful endeavor and in some ways this has been answered though work employing the collection of sperm directly from a slide (laser microdissection). However, in our hands, the number of sperm on a slide provides an indication of how to proceed with the extraction of the sample from which the slide was made. For instance, in those cases where minimal sperm are recovered, this would indicate to the examiner that a larger piece of “swab” or “cutting” should be taken to affect a recovery of sufficient DNA for successful generation of an STR profile.

As we were working with casework samples, controlled studies such as taking the same amount of a swab regardless of the sperm count were not conducted since the object of our DNA work must put the best interests of the case first - in this instance the extraction of enough DNA to

proceed to DNA profiling. These results do give a qualitative assessment which is useful for the examiner to judge the amount of swab/cutting to use.

This project took longer for us to accomplish than originally expected. We spent the bulk of our time working on the scope to make those improvements that would be necessary to offer to the community an instrument to speed the analysis of sperm slides. The relationship of counted sperm cells and DNA yield could be further explored with a more controlled study if desired.

Use of the Instrument

Through interaction with the manufacturer, the MetaSystems' software program was modified to collect information that is useful to the analyst. The home screen is used to set up slide runs, adjust lighting, monitor runs using histograms, and review slide results. There are also numerous functions such as slide searching, switching to training mode, and manipulating the scope stage. Figure 4 depicts the Slide Setup screen. Here the analyst enters the slide name and chooses the run program (i.e. classifier) and number of items to be counted. The number of items to be counted can be changed as needed and will include debris and any possible sperm cells. If the number is too low the microscope may only detect a few false positives and then stop the run when only a portion of the smear is searched. Therefore, the number is usually set high enough (i.e. 2000) to ensure that the entire smear is searched. Figure 5 is an example of the main screen following a run. The slide name, "Demo2~A", is listed in yellow at the bottom-right of the screen and images can be viewed in the partial gallery. A 3-dimensional view of the slide, shown on the bottom-left, indicates that the smear on the slide was flat with no large variations in thickness. The following images show the location of each captured image (i.e. white dots) and

the red dot corresponds to the location of the first gallery image. Additional information including the run parameters (in dark blue), such as magnification (20X), number of fields viewed (746), and run time (12 minutes, 22 seconds) are provided. Other information on this screen relates to the images identified as potential sperm cells. The number 219 refers to the number of images captured on this particular slide. The green, blue, and red "0" are involved in the review process and indicate that the slide has not yet been reviewed.

The image in Figure 6 is the "Gallery" view. Similar to the main screen it shows the location, in white, of all the images captured and the red dot for the location of the image currently being viewed. The red square indicates which image is currently being reviewed and all information depicted on this page will correspond to that particular cell location. For example, the software has given this image the classifier 178 and the coordinates are 42.93 / 24.44. These coordinates can be used in the future when the slide is reloaded onto the microscope stage or if the file is reopened. As the user moves from one image to the next, the red square will also move accordingly and the pertinent information will change. If the slide remains on the microscope stage, the stage will be directed so that the analyst can view the "active" image through the objective.

The Gallery view is used to review potential sperm cells that were identified by the system during the run. Figure 7 shows the Gallery view with cells positively marked as sperm cells (images outlined in green). Alternatively, the red X's indicate images that were deemed not to be sperm cells by the analyst reviewing the results. The blue X's indicate images that have been rejected as sperm cells. This function can be used to expedite the review process for the co-reader of a slide run. For instance, the run can be reviewed by more than one examiner and

markings used to record their assessments as to which image is a sperm cell. The number 19, in green, indicates that 19 images have been positively identified to contain sperm cells out of the 173 images identified by the computer. It must be noted that there may be more than 19 sperm images present, but the examiner may have found that number to be sufficient for the review. The second image in the Gallery has green corners indicating that it has been marked as positive for sperm cells, but also has the red border showing that it is the active image being viewed by the analyst. Therefore, the coordinates 41.61 / 26.89 are specific for that image. All of the images in this Gallery can be viewed one at a time by scrolling down using the arrows on the far right of the screen.

Figure 8 depicts a single sperm cell identified along with a number of false positives. There are numerous red nuclei within the epithelial cells which the computer has identified as possible sperm cells. As the sperm cell image is selected by the software, the coordinates of that sperm cell are shown. Typically, the software sorts the images; those that have been assessed by the software as the best candidate sperm cells are the first images of the gallery (Figure 9). However, that did not happen in this case possibly because the sperm cell is slightly misshapen, having a partially broken tail section. Figure 10 also shows a single sperm cell identified on a smear. The sperm cell is slightly elongated with a small piece of debris but was clearly identified as a sperm cell by the software. Other odd shaped debris was falsely identified by the computer as sperm cells leading to a high number of images (1337) pending review. However, review on the computer screen is a relatively quick process and as mentioned previously, the software usually sorts the images based on their potential as sperm cells. Lastly, Figure 11 is an example of a slide run where no sperm cells were present. Although the software identified 38 possibilities, the reviewer deemed them all as false positives. The images were not marked with

red or blue X's as this function is usually skipped and replaced with a more efficient practice of marking positives for review.

In order to review findings from a previous run, the slide name can be easily searched as shown in Figure 12. Once the slide name is highlighted, the analyst can chose to "load file" and the corresponding slide information and gallery images will load onto the Main and Gallery screens. This process is useful if the slide information needs to be reviewed, but the actual slide is no longer available. However, as mentioned previously, the computer memory is rapidly expended because of the large file size. To remedy this, either an external hard drive is required for long term data storage or the older slide data must be deleted from the system.

B. Tables

Case #	Item #	Manual Search Result	Metasystems Scope Result	Time (min:sec)	Comments
09-BU-20902	A12-1-2-1	occasional heads	26 heads, 90 debris	06:59	
09-BU-20902	A12-2-2-1	2 heads	2 heads, 9 debris	05:17	
09-B1-01742	B1-1-1-1	1 head	1 head, 64 debris	11:48	
09-B1-01742	B1-2-1	no sperm found	0 heads, 9 debris	06:33	
09-BU-19304	A1-4-2	no sperm found	0 heads, 161 debris	20:17	
09-BU-19304	A1-4-1-1	no sperm found	1 head	33:34	possible head but hard to verify
09-C1-03094	A1-4-2	occasional heads	11 heads, 128 debris	18:46	
09-C1-03981	A1-2-2	no sperm found	1 head	27:55	possible head but hard to verify
09-C1-03981	A1-4-2	no sperm found	0 heads, 41 debris	19:35	
09-C1-03981	A1-2-1-1	1 possible head	2 heads	30:21	
09-BU-21280	A1-3-1-1	no sperm found	0 heads, 21 debris	30:29	
09-BT-02238	A1-10-1	no sperm found	0 heads, 15 debris	25:13	
09-A3-03718	A1-2-2	many heads & intacts	many heads, 429 total items	30:32	
09-A2-04297	A1-2-2	many heads & intacts	many heads, 1023 total items	33:12	
09-HF-07289	A1-3-1-1	no sperm found	0 heads, 73 debris	19:56	
09-NP-02091	A2-2-1-1	no sperm found	0 heads, 101 debris	22:07	
09-BU-17872	A1-1-1	no sperm found	0 heads, 191 debris	26:12	
09-SJ-03285	A1-2-2	occasional heads	7 heads, 5 debris	23:54	
08BD-00985	A1-2-2	many heads & intacts	49 heads, 50 debris	09:56	
09-BN-08097	A1-5-2-1	no sperm found	0 heads, 118 debris	21:08	
09-BN-08071	A1-2-1-1	no sperm found	0 heads, 32 debris	11:31	
09-ST-01771	A1-2-1-1	no sperm found	0 heads, 93 debris	24:13	
09-SB-08576	A1-4-1-1	no sperm found	0 heads, 76 debris	16:52	
09-BU-27894	A1-3-1-1	no sperm found	0 heads, 101 debris	23:07	
09-BU-26801	A1-3-1-1	no sperm found	0 heads, 12 debris	07:58	
09-BU-26801	A1-4-1	occasional heads	3 heads, 41 debris	13:02	
09-C1-05629	A1-2-2	1 head	0 heads, 627 debris	27:41	heavy debris on slide
09-MP-373868	A1-2-1-1	1 head	2 heads, 47 debris	19:37	
09-ES-08763	A1-7-1	no sperm found	0 heads, 57 debris	12:40	
09-MR-02731	A1-1-2-1	no sperm found	0 heads, 57 debris	14:30	
10-A2-00392	A1-3-2	no sperm found	0 heads, 58 debris	31:24	
09-A1-02583	A2-1-1	2 heads	1 head, 128 debris	25:25	
09-D2-03367	A3-6-2-1	2 heads	2 heads, 59 debris	20:12	

Table 1. Example of chart showing results for each automated slide search.

C. Figures

Figure 1 – Automated microscope system from MetaSystems.

Figure 2 – Automated microscope system from Loats Associates, Inc.

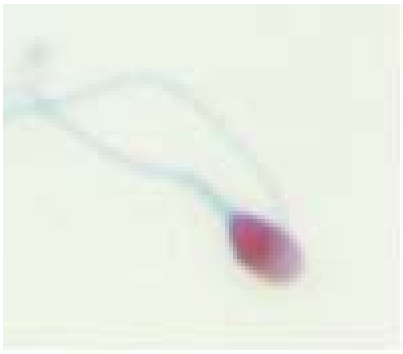

Figure 3 – Enlargement of a spermatozoon stained with Christmas tree stain.

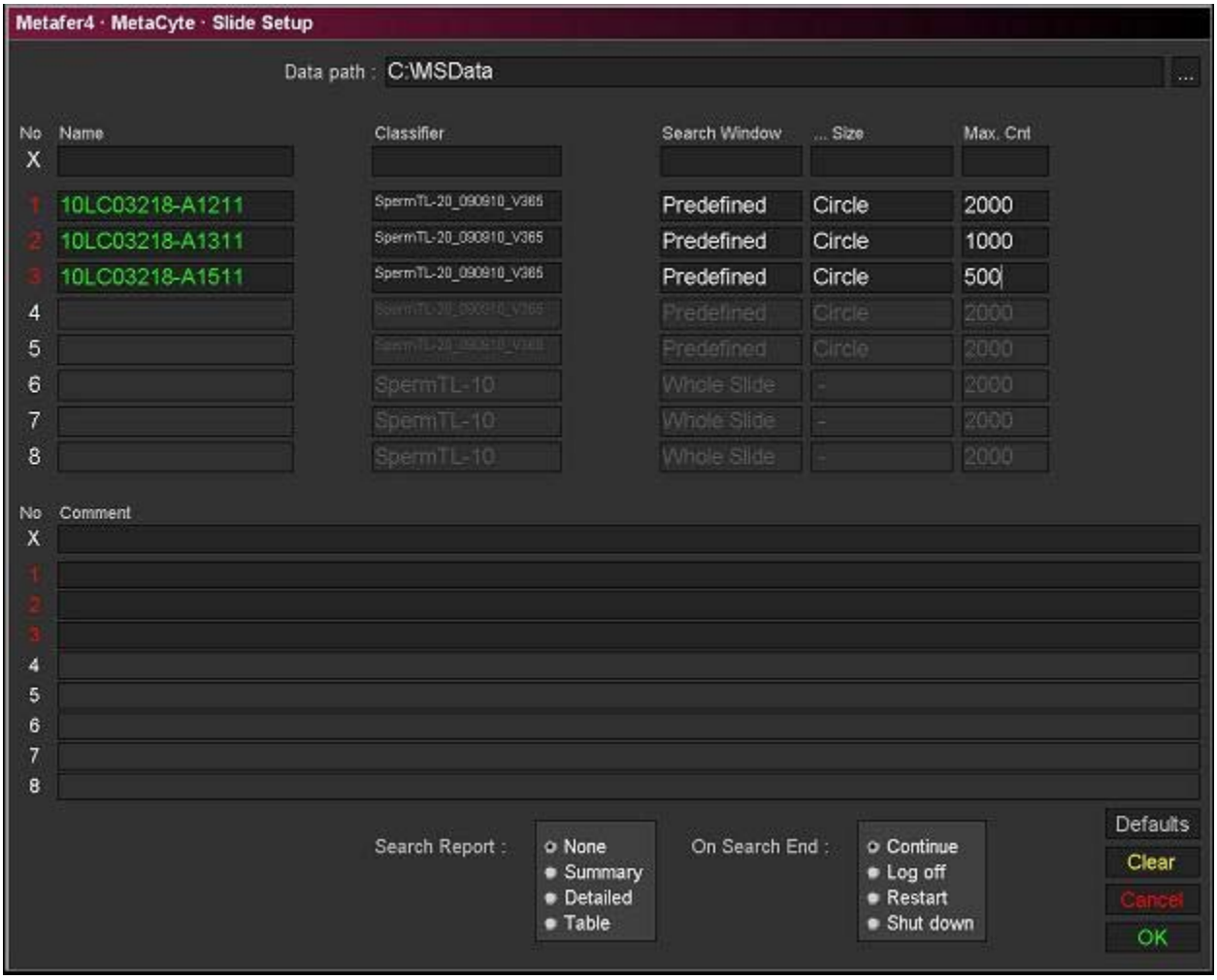

Figure 4 – Example of information required prior to an automated slide search (i.e. Slide Setup Screen).

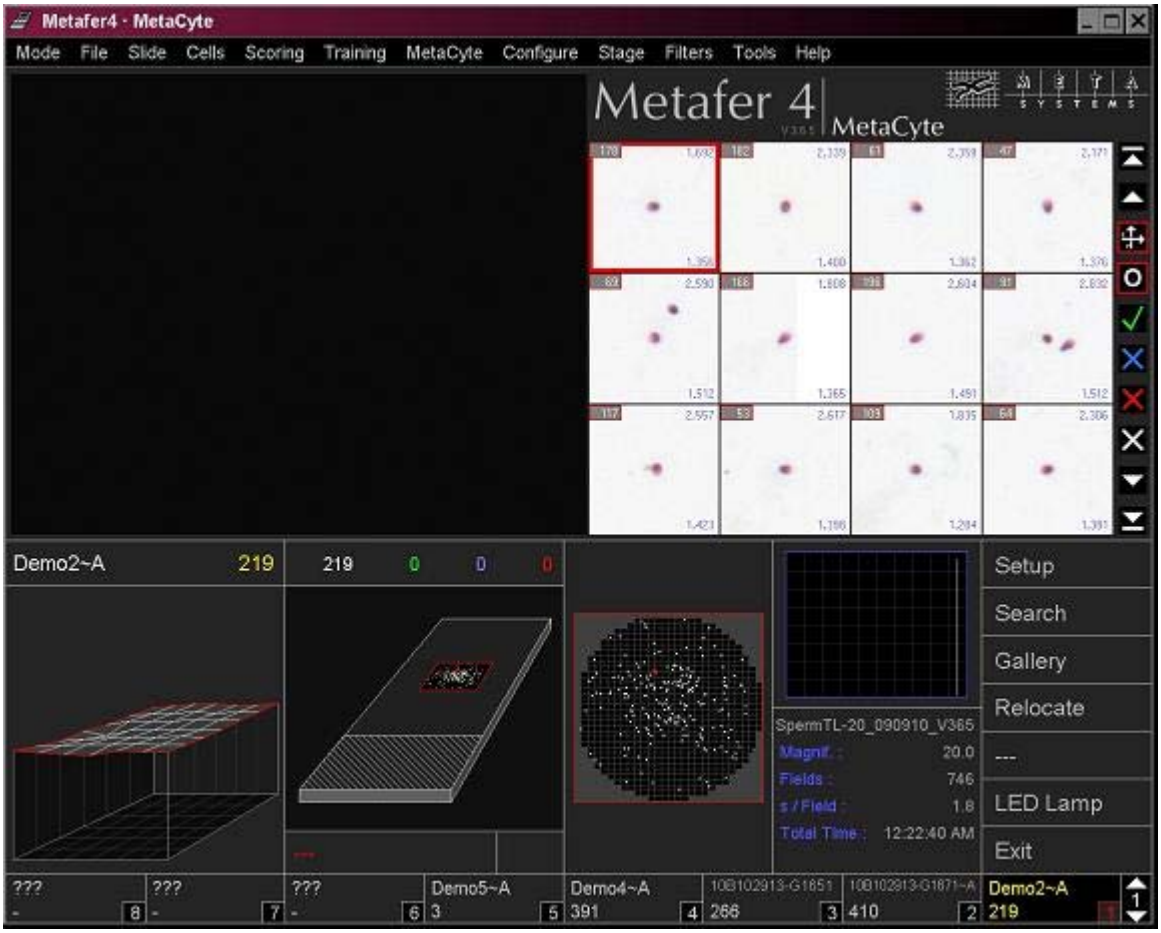

Figure 5 – Example of information provided by MetaSystems software following an automated slide search (i.e. Main Screen).

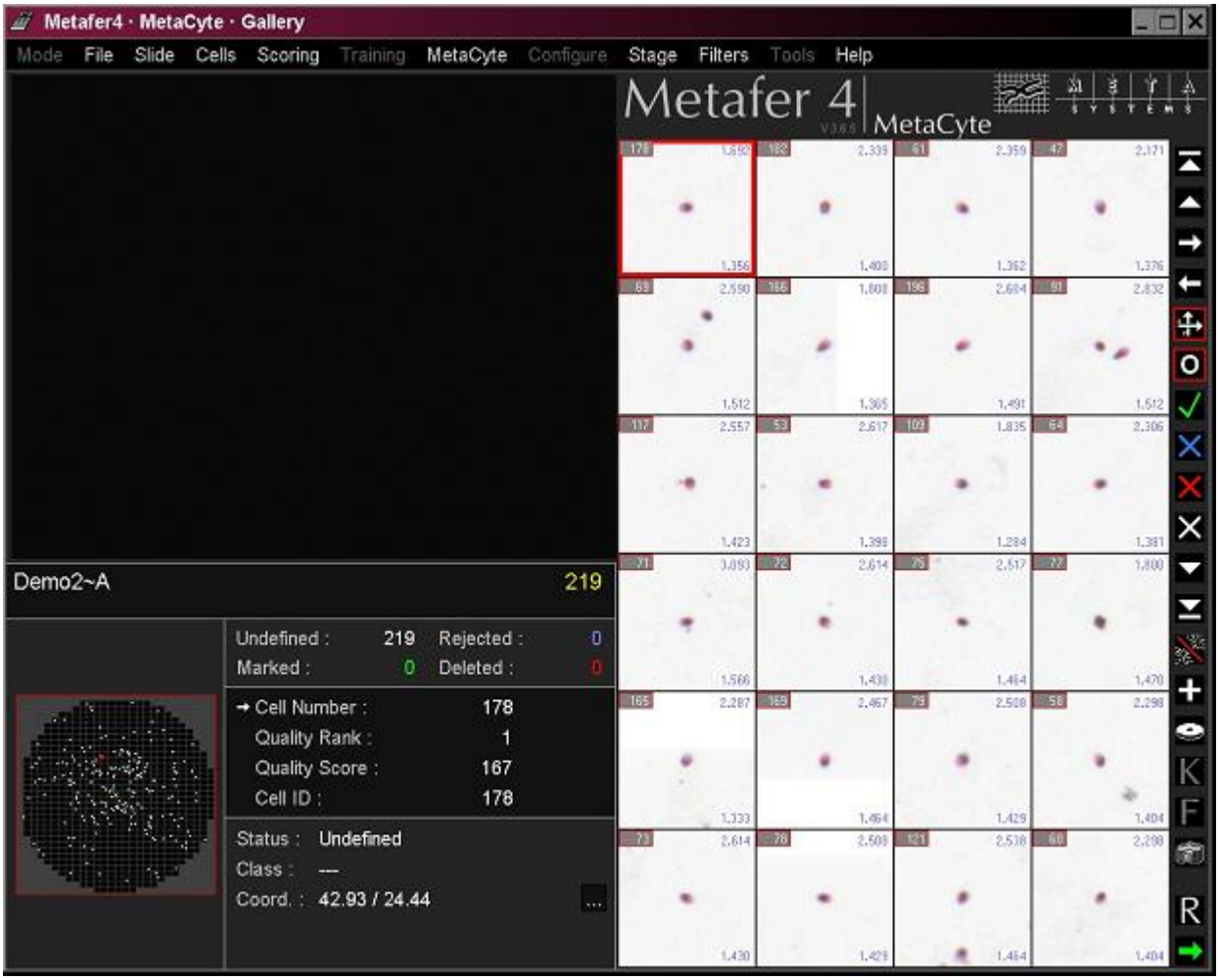

Figure 6 – Example of information provided by MetaSystems software following an automated slide search (i.e. Gallery Screen).

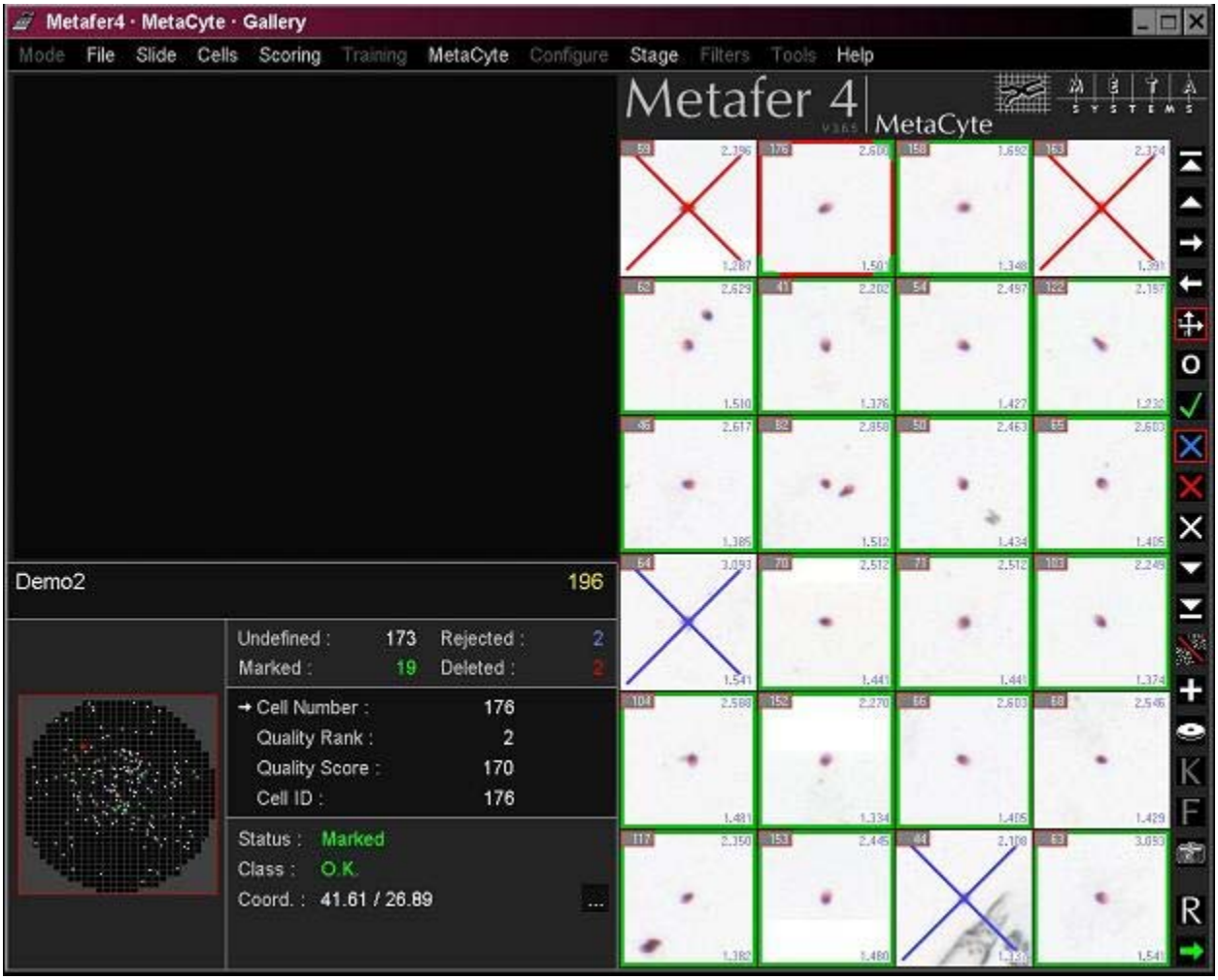

Figure 7 – Example of review process using Gallery information provided by MetaSystems software following an automated slide search.

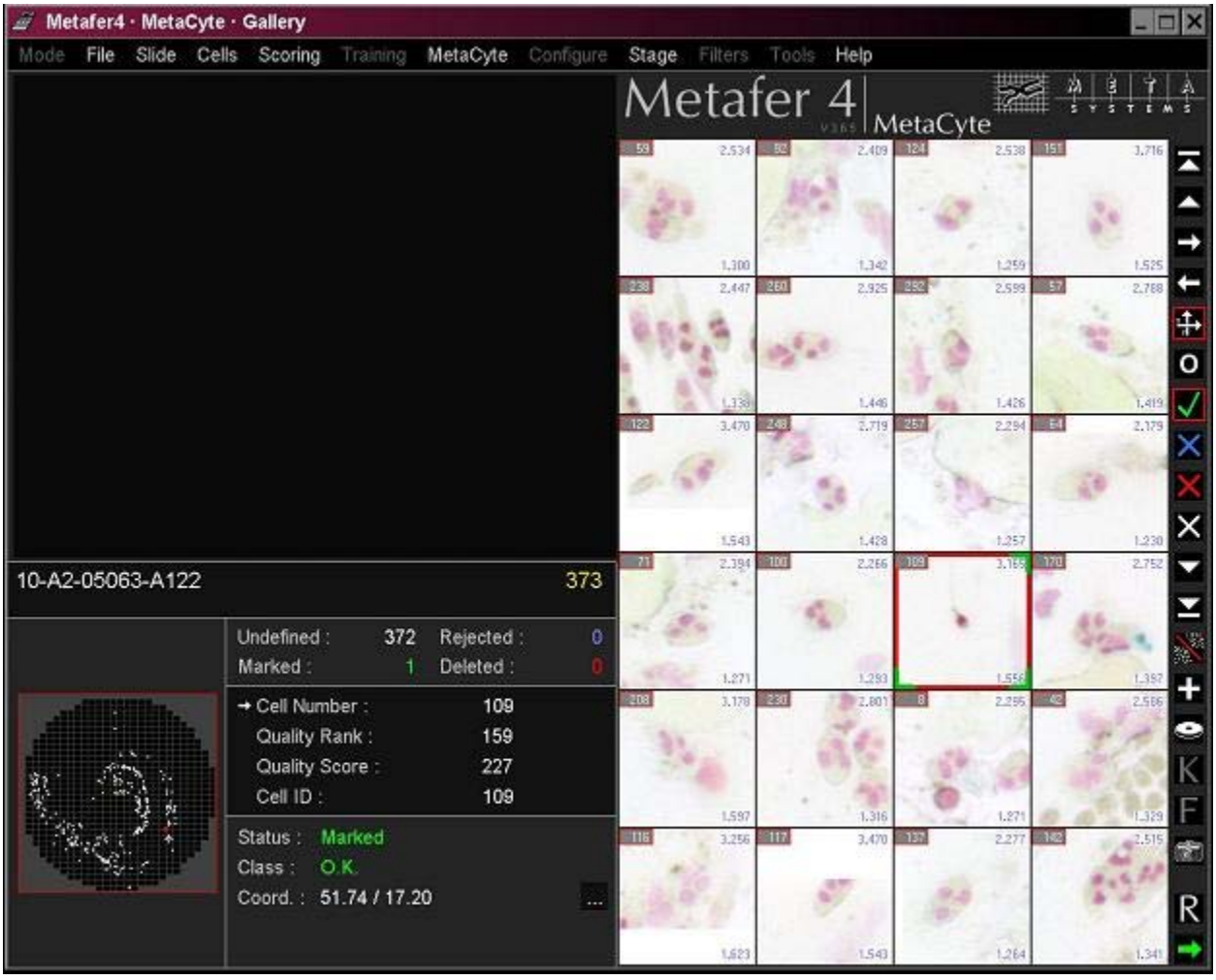

Figure 8 – Example of a positive sperm cell surrounded by false positives following an automated slide search.

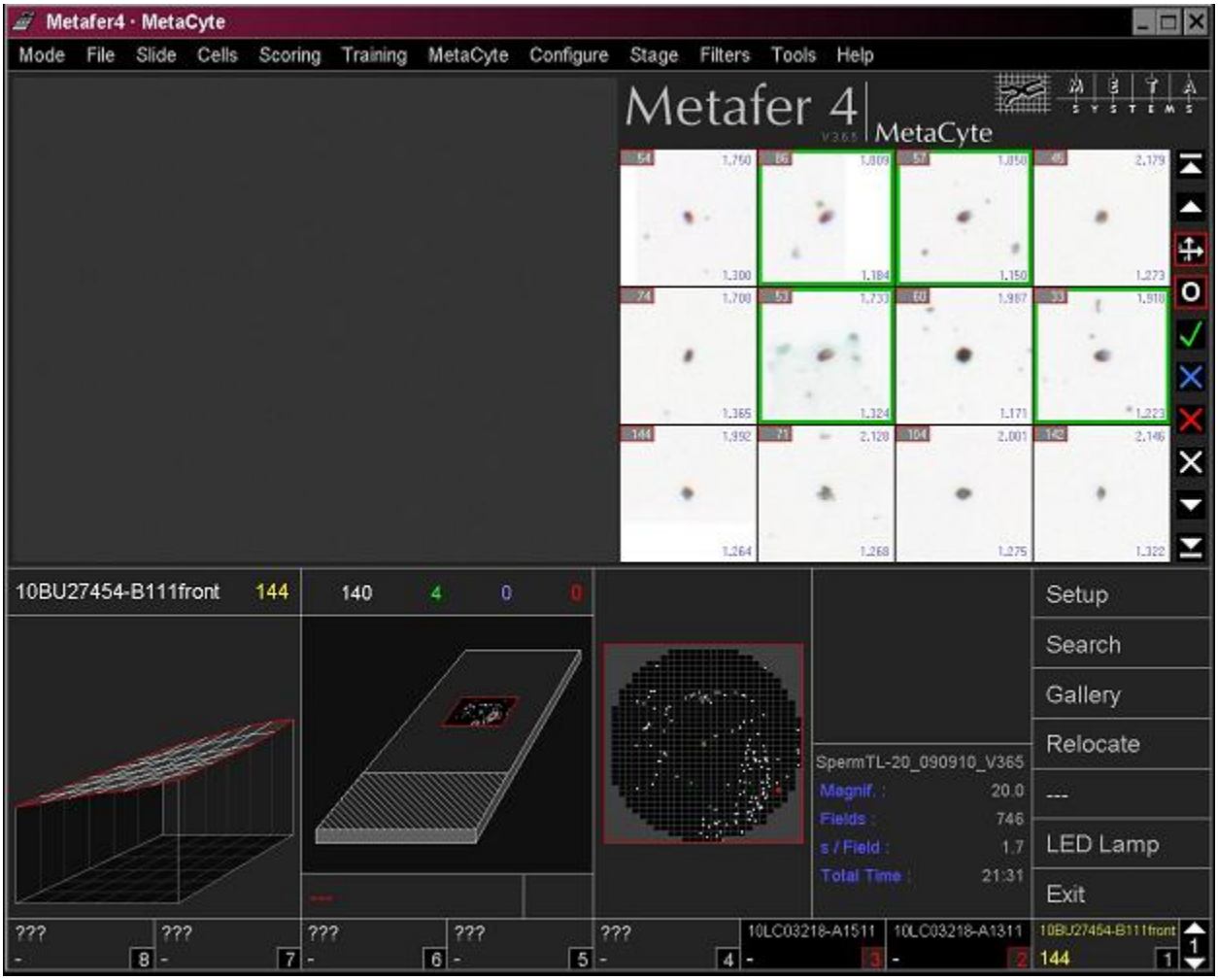

Figure 9 – Example of sorting function performed by the software following an automated slide search. The number in the upper left corner of each gallery image is the number relative to the order in which the item was detected during the scan. Here it can be seen that the numbers are not in numerical order because of the sorting performed.

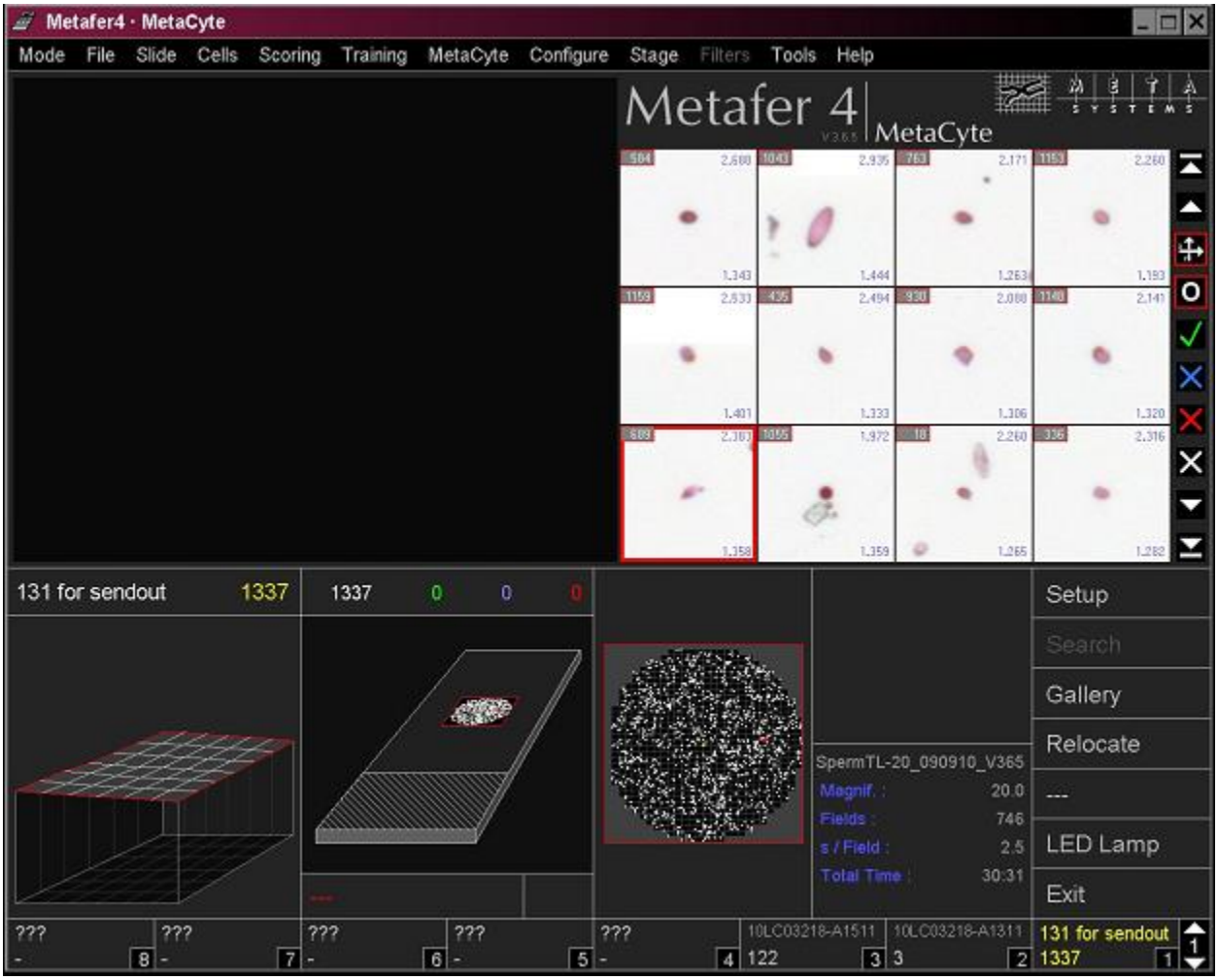

Figure 10 – Example of a single sperm cell identified on a smear following an automated slide search.

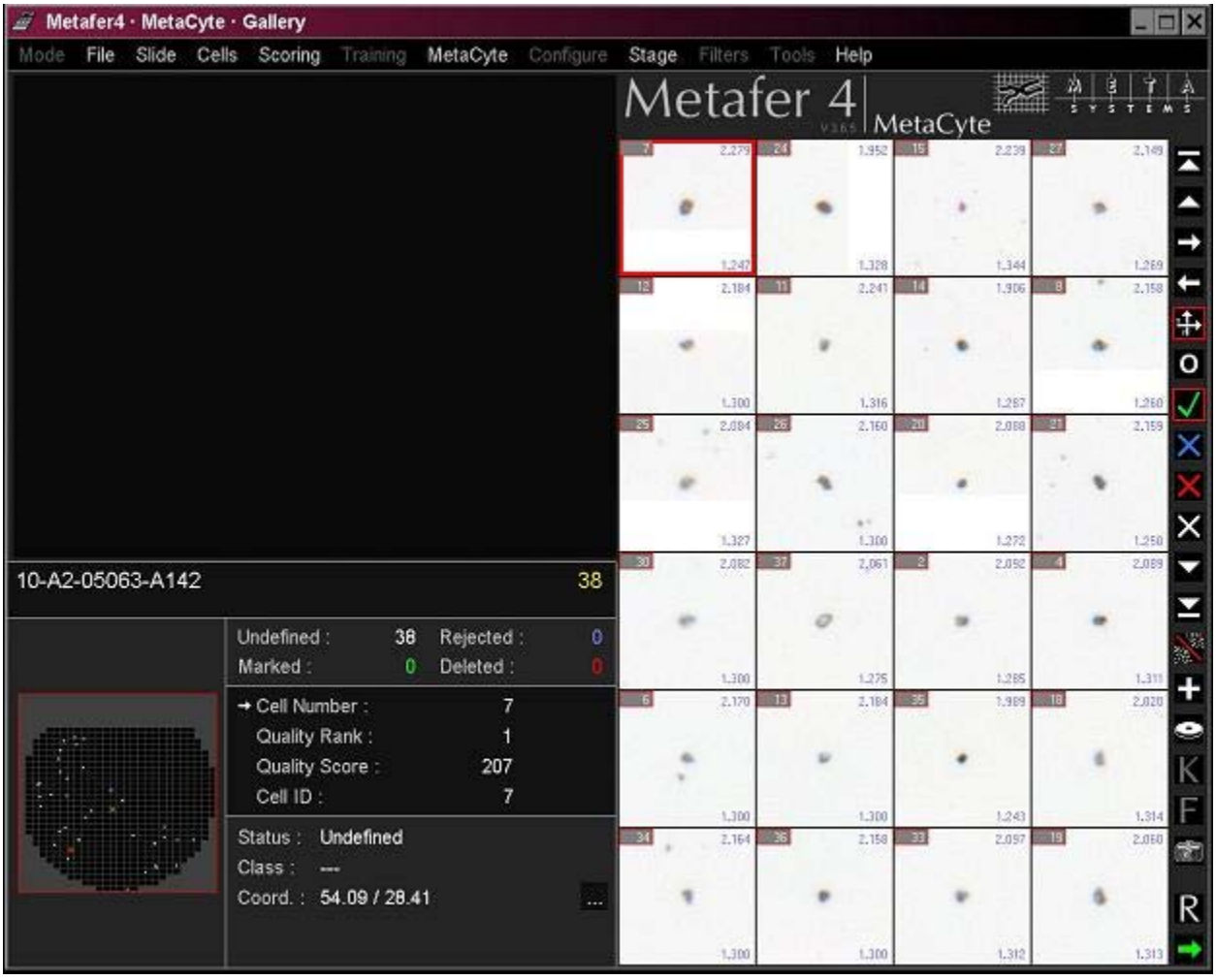

Figure 11 – Example of an automated slide search for which no sperm cells were positively identified by the reviewer.

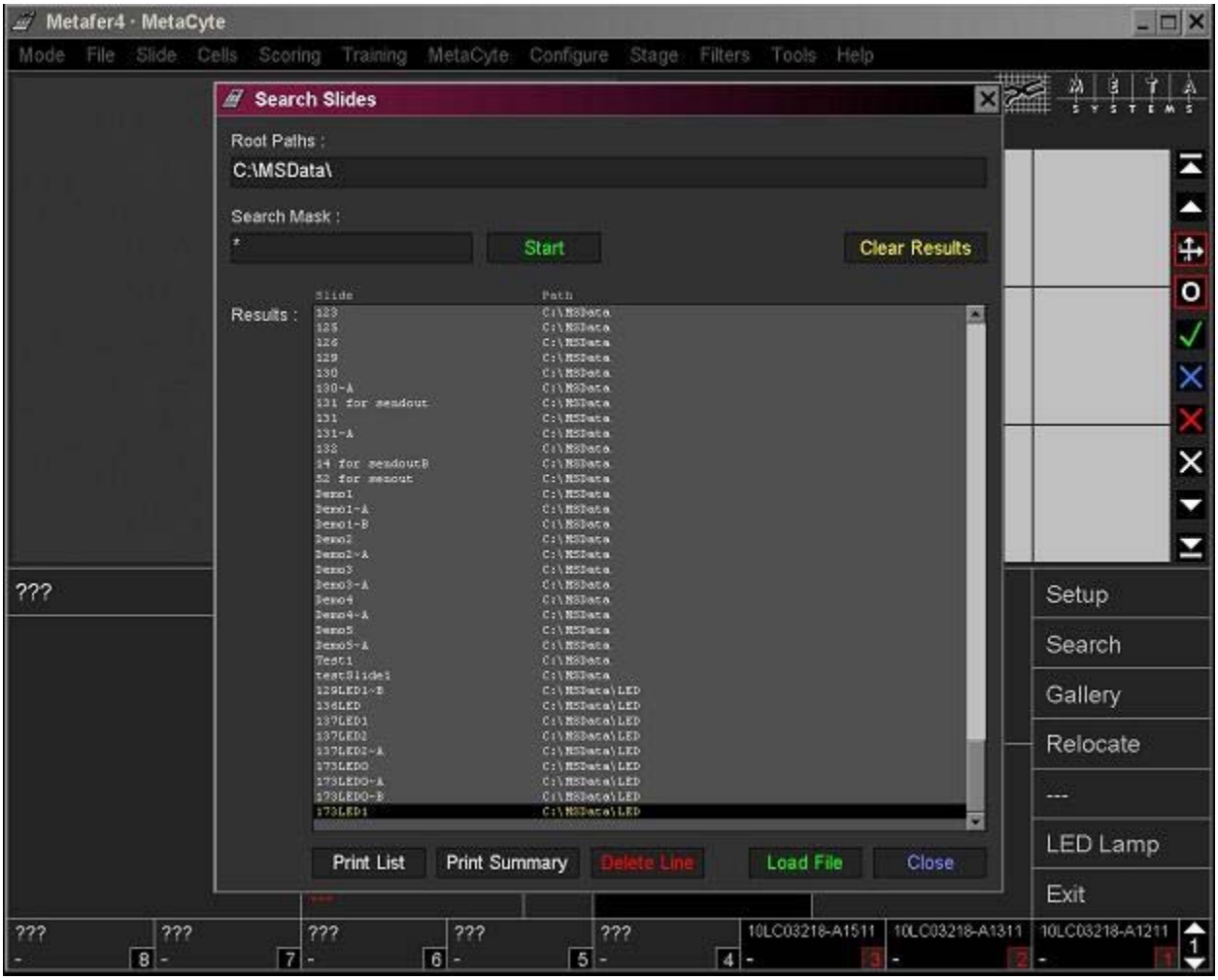

Figure 12 – Example of how to load a slide from a previous run.

IV. Conclusions

A. Discussion of Findings

The main goal of this study was to develop a fast, efficient, and reliable microscope that could be controlled by a computer software program with limited interaction from the forensic analyst to identify sperm cells on microscope slides. Fulfilling this goal would allow the analyst more time to perform other duties while the laboratory slides were being examined. In turn, this would increase the efficiency of the forensic laboratory and allow for faster conclusions of casework, thus reducing the case backlog. Also successful implementation of automated slide-reading would reduce ergonomic injuries for the analyst by decreasing the repetitive stress of manually viewing slides.

The MetaSystems microscope system is now being used in the Vermont Forensic Laboratory by all the Serologists and is considered to be extremely useful in casework. The microscope slides are easy to load onto the microscope's stage platform. The computer program is quick to set up involving only a few data entry steps for capturing casework information. While the slides are scanning, the examiner can do other hands-on tasks. Depending on the workflow for the day, the slide can be read first either manually or by the automated system. The typical process is to first use the automated microscope; if sperm cells are found, the slide can be co-read by another analyst while on the automated system. Alternatively, if no sperm cells are found, the negative slide is viewed manually. If the MetaSystems scope is in use, the manual method may be performed first, but is usually just a quick scan of the slide. If only a few sperm cells are found (referred to as "rare heads"), the automated system may then be used to get an accurate count of

how many sperm cells are on the entire smear. If no sperm cells are found during the manual search, the automated system is used to ensure that the examiner did not miss any sperm cells.

To date, the MetaSystems automated microscope has scanned 376 casework slides, either hospital slides or slides made in the laboratory. Three of these slides were found to be false negatives; a sperm cell was found manually on each slide that had been missed by the automatic scope. Of all the slides found to be negative through a manual search, none of these have then been found to contain sperm cells by the automated system. In conclusion, use of the system has greatly reduced the ergonomic problems of extended manual slide review and has often decreased the time taken to determine if seminal fluid is present on an item of evidence.

One portion of this grant was to examine whether fluorescent labeling of cells could be incorporated as a means to increase the speed of screening potential sperm cells. We went to great lengths to determine a staining procedure that would work well in conjunction with the automated sperm search process. Although this arm of the research study proved unsatisfactory and was discontinued, the efficiency savings we achieve through use of the automated sperm search procedure is significant.

B. Implications for Policy and Practice

Cases involving sexual assault make up the bulk of the biological casework done in most forensic laboratories. Thousands of sexual assault cases lie in storage rooms awaiting analysis, as victims wonder about results and assailants remain free to commit another violent crime. From the examination of the sexual assault kit to the inspection of clothing and other crime scene

evidence, the analysis of sexual assault evidence is focused on the detection pertinent of biological material. Biological material identified during this segment of the analysis is submitted to the DNA section for profiling. DNA profiling methods have drastically improved in the past ten years and major developments are being implemented to automate the profiling process. Robots have been used in a number of forensic laboratories to extract DNA from crime scene samples. The robotic dilution of extracted DNA for the purpose of quantitation is also being conducted. New, faster DNA quantitation procedures are in place. High sample volume capillary electrophoresis instrumentation is being employed and validated for crime scene sample analysis. In addition, expert systems that assist in the analysis of DNA profiles speed DNA analysis. The infrastructure to allow DNA profiling to proceed quickly and efficiently is slowly being realized. Considerable time and resources have been devoted to this segment of the analysis and it is beginning to pay off. It is now time to look at how we can efficiently process samples destined for the DNA unit.

The biological examination of evidence has not changed appreciably over many years. Some manual tests have become faster and easier to perform, however little automation exists to increase the productivity of the analyst who examines sexual assault type evidence. The introduction of an instrument to the forensic community that is designed to automate the examination of sperm slides would be a major advance to expedite the processing of sexual assault evidence. Steps that allow an expeditious evaluation of the large number of backlogged kits would be of tremendous advantage to the criminal justice community. Analysts could allow this instrument to process slides while pursuing other examinations. One could imagine a carousel filled with slides that are processed automatically. Those slides with sperm cells could be quickly reviewed by an analyst with directed viewing by the instrument. Positive cases could

be further processed for DNA. Negative cases could be reported in a timely manner to officers to allow further investigations to proceed, with the knowledge that DNA would not be of assistance in those particular cases. With appropriate research, one could determine the likelihood of DNA profiling results based upon the number of sperm observed. The ability to determine which samples would have a high probability of success would save time and resources in the DNA unit.

C. Implications for Further Research

Ideas to pursue in future research would be the development of a standard set of slides that are fixed in such a way so that the stain intensity does not fade over time. This would serve as a quality control check of the autoscope before use, similar to other instruments in the forensic laboratory setting. Another improvement could be the use of the printer so that an image of the cell could be captured and a high quality photograph printed out for inclusion with the casework paperwork. Lastly, the microscope's computer system could be networked to the laboratory information management system (LIMS) so that the cell image could be saved to an electronic case file.

V. References

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VI. Dissemination of Research Findings

While the system will be useful in our own laboratory, a major goal was to distribute this technology to the forensic community at large to improve criminal justice in the United States. To this end, we took a multi-tiered approach at disseminating our work. The first step was talking about our progress at scientific meetings with included the NIJ Grantees meeting and NIJ Conference.

Presentations at Scientific Conferences:

National Institute of Justice Seventh Annual DNA Grantees Workshop, Arlington, VA, June 2006; "Demonstration of an automated microscope for the identification of sperm cells", Demonstration

National Institute of Justice Conference, Arlington, VA, July 2010; "Demonstration of an automated microscope for the identification of sperm cells", Demonstration

One approach to disseminating our results was to work with a company to create an inexpensive as possible, user-friendly forensic sperm finder package system for sale to the forensic community. The system we created in partnership with MetaSystems is commercially available and the costs vary depending upon the accessories. At this point, the fluorescent package would not be required for operational use.

Another approach to disseminate our work was to join forces with the National Forensic Science Training Center and the American Academy of Forensic Sciences and to hold work-shops to offer a hands-on approach to the transfer of technology developed through this grant. We have been in touch with staff at the NFSTC and welcome the opportunity to hold workshops or demonstrate this unit compared to any other commercially available system.

The last way to disseminate our work was to publish our results in forensic journals. We will consider publishing our work, but it may not be viewed as novel and may be considered little more than an advertisement for the MetaSystems product. Nevertheless, we will review our data and see if this would be something worthy of publication. We have also posted our willingness to demonstrate our unit to a forensic user group, and we have demonstrated the unit to several interested parties (forensic labs in New England and the RCMP who eventually purchased the MetaSystems unit).

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