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Protocols for Automated Protist Analysis

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16. Abstract (MAXIMUM 200 WORDS)

Ballast water management systems must be tested relative to the U.S. Coast Guard's proposed ballast water discharge standard before they can be approved for routine shipboard use. Analysis for live organisms in treated samples is time-limited and labor intensive. Currently, skilled staff must use a microscope to observe, count, and verify the viability of live organisms ≥ 10 μm and < 50 μm in minimum dimension. This program built upon previous work performed by the Naval Research Laboratory to develop data management, data archive, and data analysis protocols for counting and determining the viability of organisms in this size range. An automated method was sought to significantly reduce staff time and effort, maintain consistency (by reducing human error and bias), and provide an archive of analytical results. Commercially available laboratory equipment and computer software were used in conjunction with specially developed pattern recognition software. This report provides a description of the equipment required and the protocols developed.

This report's findings are the result of continued research into the potential to automate protist analyses and provide the most current information available at the conclusion of this initial research effort. Additional automation research efforts are ongoing and may affect details reported herein.

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EXECUTIVE SUMMARY

Ballast water is a known pathway for the introduction of aquatic nuisance species (ANS). In an effort to reduce the number of introductions of ANS into United States waters, the U.S. Coast Guard (USCG) has proposed regulations requiring ships to meet stringent discharge standards. The proposed concentration for organisms ≥ 10 microns (μm) to < 50 μm in minimum dimension (nominally protists) is less than 10 living organisms per milliliter of ballast water. This concentration level may later be reduced (i.e., a more-stringent standard, Phase II, may be enacted) if USCG determines ballast water management systems (BWMSs) can meet the more stringent standard and doing so would be practicable. Before the USCG can approve BWMSs for routine use aboard ships, the BWMS vendor must demonstrate they are capable of meeting a given discharge standard.

A protocol for testing BWMSs at full scale has been developed by the Environmental Protection Agency's Environmental Technology Verification Program in cooperation with the USCG. Among other metrics, testing requires evaluating treated samples to determine the number of living organisms in the 10 to 50 micron (≥ 10 μm to < 50 μm) size class. Current manual methods require skilled personnel using microscopes to observe, enumerate, and determine the viability of organisms in concentrated samples before mortality is shown to occur from the artificial conditions of holding samples. This visual analysis is labor intensive, requires skilled personnel, is subject to operator fatigue, and provides no archive of results. The USCG Research and Development Center therefore sought a method to automate analyses of organisms in the ≥ 10 μm to < 50 μm size class.

Recent Naval Research Laboratory (NRL) research efforts have shown that pattern recognition algorithms could be applied to sequential photomicrographs of treated samples of organisms ≥ 50 μm to identify motion and, therefore, the viability of organisms. These motility algorithms have also proven to be applicable to the analysis of organisms ≥ 10 μm to < 50 μm samples during recent work conducted with concentrated natural seawater samples. In addition, the use of a sample staining procedure has helped in the identification of non-motile viable organisms in the ≥ 10 μm to < 50 μm size class. Subsequent work refining algorithms, improving equipment, and investigating appropriate vital and mortal stains led to a practical protocol that could be used routinely during tests of BWMSs.

This report provides background information on how the automation protocol was developed, describes the type of equipment used, and presents the protocol for use by other test facility operators. The text discusses setting up data archives and image collection. The basic protocol is discussed in the text, and a step-by-step protocol for routine laboratory use is provided in an appendix.

Results of this research effort will provide consistent analyses of organisms in the ≥ 10 μm to < 50 μm size class in treated ballast water samples with a significant reduction in skilled staff-hours required. The government-developed protocol will be available free of charge in the public domain.

The findings in this report are the result of continued research into the potential to automate analyses of organisms ≥ 10 μm to < 50 μm and provide the most current information available at the conclusion of this research effort. The emphasis of this year's effort was processing the types of samples that would be encountered during the standardized testing of BWMSs. Additional automation research efforts are ongoing and may affect details reported herein.

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LIST OF ACRONYMS, ABBREVIATIONS, AND SYMBOLS

ANS	Aquatic nuisance species
BWMS	Ballast water management system
CMFDA	5-Chloromethylfluorescein diacetate
CCD	Charged coupled device
COTS	Commercial Off the Shelf
DIA	Diascopic (brightfield illumination)
FDA	Fluorescein diacetate
GFP	Green fluorescent protein
GUI	Graphical user interface
m^3	Cubic meter
mL	Milliliter
mm	Millimeter
ms	Millisecond
ND	Neutral density
NRL	Naval Research Laboratory
NRL KW	Naval Research Laboratory in Key West, Florida
PC	Personal computer
RGB	Red green blue
s	second
SR	Sedgwick Rafter (counting chamber)
X	Magnification power
µL	Microliter
µm	Micrometer (micron)
<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
.nd2	Image file type
.xls and .xlsx	Excel Spreadsheet file type

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1 BACKGROUND AND INTRODUCTION

Testing to evaluate the efficacy of a Ballast Water Management System (BWMS) requires the characterization of samples to determine the number of live organisms after treatment. Standardized BWMS testing requires that organisms be characterized in three size classes based on maximum dimension on the smallest axis: organisms ≥ 50 microns (μm) (nominally zooplankton), organisms ≥ 10 μm to < 50 μm (nominally protists), and organisms < 10 μm (here, culturable, aerobic, heterotrophic bacteria) (U.S. EPA 2010). The Naval Research Laboratory (NRL) recently published protocols for the collection of image sets, test documentation, image set analysis, and data archive for organisms in the ≥ 50 μm size class (Nelson, et al., 2010b; Nelson, et al., 2011). These documents additionally describe the equipment and software required to implement this sample analysis protocol for organisms ≥ 50 μm. This protocol has also proved useful for the data collection, analysis and archive BWMS sample data for organisms in the ≥ 10 μm to < 50 μm size class. The major differences between the two protocols for these two size groups are: 1) the specific volume of samples that are analyzed, 2) the size of the micro-wells or Sedgwick Rafter (SR) counting chambers that are used when collecting microscope data, and 3) the total microscope magnification required to image the samples.

With respect to organisms in the ≥ 10 μm to < 50 μm size class, Phase I of the U.S. Coast Guard (USCG) proposed discharge standard and the International Maritime Organization (IMO) discharge standard require that there be less than 10 living organisms per milliliter (mL) (U.S. Federal Register, 2009). Statistical analyses suggest that a sample for this concentration of viable organisms, 3-6 liters (L) of sample should be concentrated to 1L. From this concentrated sample, two to four 1-mL samples must then be analyzed if a coefficient of variation not greater than 10% is desired (EPA, 2010). To keep organisms slightly larger than 10 μm in focus in a sample, the typical individual sample volumes that will be analyzed are 20 microliters (μL), which necessitates that between 100 and 200 individual micro-wells are analyzed to determine if a sample is compliant with discharge standards. For samples with sparsely distributed organisms (such as treated ballast water), higher concentration factors may be necessary to minimize the required analysis volume.

Because standardized tests also require that suspended solids and other water properties (e.g., mineral matter and dissolved organic carbon) fall within specified ranges, concentrating samples three- to six-fold (EPA, 2010) will also increase the amount of suspended solids in the same general size range as the organisms being assessed. This process significantly increases the complexity of samples since organisms can be obscured from view by sample debris. The requirement to analyze a large number of samples, combined with the complexity and diversity of organisms in the ≥ 10 μm to < 50 μm size class (along with the addition of suspended solids in this same size-range), makes it challenging to accurately characterize samples using manual microscopy methods. The motility of organisms in the ≥ 10 μm to < 50 μm class is an additional concern. Although many organisms in this size class are relatively non-motile, others are extremely motile and can move across an entire micro-well in seconds. Further, suspended solids in the sample can allow organisms to "hide" or be obscured by debris during observation. Given the motility of some organisms, it is critical that the entire micro-well is visible when conducting analyses or that organisms are tracked as they enter and leave the microscope's field of view. The alternative approach, magnifying selected regions of the field of view, provides information regarding only those organisms in the magnified region, which can result in organisms being under or over counted.

The requirement to observe the entire micro-well or an area that is much larger than typical organisms can be addressed using a microscope and camera system with sufficient spatial resolution that is matched to a

micro-well or counting chamber. When these systems provide adequate spatial resolution, it becomes feasible to "zoom in" on the individual organisms in the digital image set after the data has been collected from the entire micro-well. Our work indicates that motility is important to determining the viability of organisms in the ≥ 10 μm to < 50 μm, thus time-resolved images are collected, and motility algorithms are directly applied to these image sets.

In an effort to find a method suitable for automation, NRL convened a workshop in January 2008 to compare multiple methods of determining viability in the ≥ 10 μm to < 50 μm size class. Statistical analysis of the multiple experiments indicated that although there were significant differences between the methods, no one method could be considered superior to all others (Nelson et al., 2009; Steinberg et al., 2011c). As a result of this workshop, NRL pursued the development of additional methods using manual microscopy.

NRL explored a variety of biological "vital stains and mortal stains" to determine the viability of protists in the ≥ 10 μm to < 50 μm class. During this effort, NRL stained ambient plankton samples as well as algal monocultures with numerous biological stains. Results of over 100 trials showed that no single stain or combination of stain adequately stained all organisms. Stains evaluated included Neutral Red, Aniline Blue, CellTracker™ Green (CMFDA), CellTracker™ Blue, SYTOX Green, SYTOX Orange, Calcein AM, and Fluorescein Diacetate (FDA). Although no single stain or combination of stains provided perfect results across all samples analyzed, the combination of two vital stains provided more consistent results than all of the others: Fluorescein Diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA, CellTracker™ Green; both stains from Molecular Probes-Invitrogen; Carlsbad, CA; Steinberg et al. 2011b). FDA and CMFDA are added to samples at a final concentration of 5 micromolar (μM) and 2.5 μM, respectively. After entering living cells, non-specific esterases in the cell cleave these stains, resulting in a molecule that fluoresces green when excited with blue light. Work performed at NRL indicated that by using a combination of these two stains, the viability of a greater number of organisms can be determined compared to using these stains individually. This work also demonstrated that many organisms' fluorescence signals can be observed even when an organism is obscured by debris.

In addition, NRL has identified a Commercial-Off-The-Shelf (COTS) micro-well that is suitable for the analysis of 20 μL samples that when completely imaged provides adequate resolution for the detection of organisms ≥ 10 μm in size. These are the SensoPlate Plus™ 384 (Greiner Bio-One; Monroe, NC). Although adequate, some spatial distortions in the image data have been observed using these well plates. These distortions are the result of misalignment of the microscope system. As a result of these observations, NRL has also looked at alternative approaches for holding samples under microscopes. At this time, based on our current laboratory efforts, NRL plans to use standard volume unruled Sedgwick Rafter (SR) counting chambers with the microscope magnification adjusted such that a 20 μL volume is imaged. This approach affords image sets of superior quality compared those obtained using micro-well plate trays. Further, this approach is less expensive and more accessible than pursuing custom low volume (20 μL) SR counting chambers with multiple counting wells housed on each counting chamber for holding samples. Laboratory work performed to date provides a strong indication that this will provide an adequate and cost effective means for imaging samples in the ≥ 10 μm to < 50 μm size class. It does, however, create requirements that organisms be tracked as they enter and leave the microscope's field of view.

This year's effort focused on working with samples representative of those are encountered during BWMS evaluations. This work included samples representative of those associated with tank filling operations, control tank drain operations, and test tank drain operations. Significant effort has been made to mimic the properties of typical samples from each of these types of operations including expected concentrations of live organisms.

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2   APPROACH

Under this program, NRL developed protocols for the collection, documentation, analysis, and archiving of image data to support BWMS evaluations. This report will first describe the equipment and software required to collect data according to these protocols. This is followed by descriptions of the laboratory procedures used to collect and analyze image data according to these protocols. If other laboratories involved with BWMS testing use the equipment and software specified in this report, then the protocol described here should find broad application in supporting standardized testing of BWMS.

It is assumed that samples have been prepared prior to the implementation of this protocol. For organisms ≥ 10 µm to <50 µm, this requires that samples first be concentrated to ensure that a sufficient sample volume is analyzed to achieve accurate analyses of a sparse population of organisms in this size class (< 10 viable organisms mL^{-1}). The concentration factor will vary based upon sample quality, sample stage, and the type of BWMS evaluated. For example, tank fill samples with their higher populations of viable organisms ≥ 10 to <50 µm (e.g., $\geq 1,000$ viable organisms mL^{-1}) may not need to be concentrated, while treated samples with sparse populations of viable organisms may require the samples to be greatly concentrated.

Following sample concentration, the sub-samples are stained using a combination of CMFDA and FDA (Nelson et al. 2010a; Nelson et al. 2010b; Steinberg et al., 2011a, b). This staining procedure requires a ten-minute incubation period prior to sample analysis. Following staining and incubation, image data can be collected for a maximum 20 minutes. Using automated analyses, this period should be sufficient to observe at least 25 20 µL samples with the current 31-second (sec) observation windows. For organisms in the ≥ 10 µm to < 50 µm size class, we may want to extend this observation window to afford the analysis of a higher number of 20 µL samples. It should be noted that NRL continues to perform work to determine if this 20-min period can be extended, which would allow a larger sample volume to be analyzed in the allowable time period.

Based on previous work, all sample concentration, staining, and image collection must be completed soon after obtaining a sample (e.g., within 6 h). Image and data analyses can be performed after this time, as they are performed on archived image sets that are collected according to this protocol.

2.1   Equipment

To characterize organisms in the ≥ 10 µm to < 50 µm size range with this protocol, two microscope modalities, i.e., brightfield (white light) and epifluorescence illumination, are required. Work performed at NRL and detailed in a separate paper shows there is an advantage to applying motility algorithms to samples and to the image sets generated from both microscope modalities. Combining these measurements allows the user to assess the viability of a wide range of organisms in the complex samples associated with BWMS evaluations. It is also necessary to be able to spatially correlate the image data from both of these modalities. Therefore, image sets from brightfield and epifluorescence must be interleaved, not collected in series.

In support of this program, NRL made additional modifications to its microscope and camera system to simultaneously collect both brightfield and epifluorescence time series image sets reliably with required image update rates. This task was accomplished using a new light source and mechanical shutter that allows the microscope to switch between brightfield and epifluorescence modes under computer control with

necessary timing precision and reliability. In this fashion, this equipment has been used to collect spatially correlated brightfield and epifluorescence image sets. To analyze samples, 64 images are collected (32 brightfield and 32 epifluorescence). To eliminate problems that frequently occur with the first images from each microscope modality, they are deleted, resulting in the collected image sets consisting of 31 brightfield and 31 epifluorescence images that were collected over a 31sec observation window for each micro-well or Sedgwick Rafter (SR) counting chamber. Reducing the observation window to afford higher sample throughput is under consideration and will most likely be advantageous for samples organisms in the ≥ 10 µm to < 50 µm size class. The benefit of reducing the sample observation window is that it will permit a larger number of 20 µL samples to be analyzed in allowable time periods before sample degradation.

2.2 Data

Data management and the documentation of system settings are critically important and assure the proper collection of image sets and archive of test results. The imaging system (which includes the microscope, camera, and the attached computer) used at NRL stores all of the image data from a given measurement into a single, large file (denoted an *.nd2 file). In addition, a spreadsheet (with embedded macros and forms) is used by the microscope operator to enter and record information specific to a given test. The operator also fills out fields in this spreadsheet to document all of the system settings. The spreadsheet and its embedded forms also provide a checklist to ensure that the operator has made all necessary system adjustments prior to data collection. This test-specific Excel spreadsheet maintains this information in an .xls or .xlsx file. Much of these same data are also stored as metadata in the test-specific *.nd2 file.

Both of these files have the same name, with two exceptions: 1) the application extension, and 2) a file name suffix with three digits (e.g., 001) to count replicate measurements from a single sample. The three-digit counter indicates that the files are collected and analyzed with the same settings. In this latter case, a numeric value is added to the end of *.nd2 file name to indicate the replicate number. Consequently, all data associated with a given test are stored in two files that have very similar names but different application extensions (i.e., .xls or xlsx and .nd2). These files are named in a manner that allows many of the test details (e.g., test date, type of sample analyzed, some system settings, and replicate or sub-sample number) to be discerned from just the file name. This procedure facilitates file recognition and retrieval when reviewing multiple tests.

Data are archived by storing files in a directory with an intuitive structure. First, the directory hierarchy separates data by the year, month, and day of collection (each is a separate directory – e.g., C:\2011\03_Mar\15). The Excel spreadsheets described above are stored directly into a specific-test directory under the day directory. Each *.nd2 file is stored in its own directory under the specific-test directory. This directory has the same name as the *.nd2 file without the application extension (described in more detail in Appendix A). The file naming and directory structure provides a means to access data associated with a given test. Further, the file-naming conventions allow important information about the test to be discerned prior to analyzing the data. Lastly, the approach used by NRL requires only two files (both automatically generated during the data collection process) to archive all settings, other relevant test information, and the test data (image sets) associated with a given microscope measurement. Many of the systems settings are redundantly archived in both of these files, providing additional corroboration of proper system settings.

Data analysis is performed using a stand-alone application developed by NRL. When launched, this application provides a graphical user interface (GUI) that asks the operator to specify the *.nd2 file to be

analyzed. The analysis application parses the *.nd2 file, performs all analyses, and stores its results in the same Excel spreadsheet that was used to document test-specific settings. The current focus of these algorithms is to enumerate the number of viable organisms in a test sample. For organisms ≥ 10 to <50 μm, viability is determined by either an organism having an epifluorescence signal or by the detection of organism motility in either the brightfield or epifluorescence image sets.

3   EQUIPMENT AND SOFTWARE REQUIREMENTS

The imaging system consists of the microscope, camera, and control hardware (i.e., a personal computer [PC]) **and** software. The overall requirement for spatial resolution for the complete imaging system is to provide at least 4 μm of spatial resolution in the microscope imagery. Since motility algorithms will be used with the samples, the entire micro-well needs to be imaged to prevent organisms from entering and leaving the field of view during the observation window (which results in counting errors). The water column depth of the sample must be consistent with the depth of focus of the microscope. If organisms in this size class move up and down through a deeper water column, they literally can disappear (as well as appear to change size). The appropriate water column depth prevents organisms from being size distorted or not observed as a result of microscope focus issues. The microscope system must also be capable of switching automatically between brightfield and epifluorescence modalities under computer control. This switching must be accomplished rapidly enough to afford a 1-sec interval between successive images that comprise the image set.

NRL has evaluated two methods for holding samples under the microscope for automated analysis of organisms ≥ 10 to <50 μm: a micro-well tray with individual micro-wells of approximately 3 to 4 mm in diameter and an unruled Sedgwick Rafter counting chambers. NRL also considered the potential of using customized unruled SR counting chambers, but the costs of developing and using this type of device could not be justified based on the potential benefits that it would provide. With any of these approaches, 20 μL is the largest sample volume that can be analyzed. In the case of the micro-well tray, water column issues primarily limit samples to this volume; that is, organisms may move out of focus if the depth of the water column is too great. In the case of SR chambers, spatial resolution issues limit samples to this volume. These sample-holding methods are discussed in detail below (section 3.1).

The USCG neither endorses nor recommends specific equipment or manufacturers. The concepts and algorithms developed in this project were developed for the specific microscope and camera systems resident at NRL. Other equipment with equivalent properties and attributes that provide the resolution and capabilities necessary to support this protocol can be used. However, many key system parameters that are currently monitored by system software may not be recorded as image file metadata with different hardware and operating software. Further, analysis routines will need to be modified to parse image data from a different source and may need additional modification if camera resolution and dynamic range are not identical to those used in the NRL systems.

3.1 Sample Holding Methods

The requirements for the microscope and camera systems are driven largely by the sample well (or other methods used to hold and analyze samples) and the dimensions of the sample container. Ideally, images of the entire sample well must be collected while providing sufficient resolution to allow organism details to be observed in the same images by changing the digital zoom level. The NRL system always provides at least 4 μm of spatial resolution when zoomed in, and the entire well is visible with all configurations under

consideration. If organisms are capable of entering and leaving the microscope's field of view, these organisms should be tracked and accounted for dynamically when generating counts from microscope observations.

Building upon work previously performed to optimize the sample wells for both organisms ≥ 50 µm and organisms ≥ 10 µm to < 50 µm, the SensoPlate Plus™ 384 (Greiner Bio-One; Monroe, NC) was considered the baseline method for working with protist samples. These wells are approximately 3-4 mm in diameter and afford spatial resolution of approximately 3 µm with the microscope optics configured as discussed in the next report section. Figure 1 provides images collected with the NRL microscope-camera system (described in the next report section) from a SensoPlate Plus™ 384 sample well. Analysis of the images indicates that these well plates when used in conjunction with the NRL microscope would provide sufficient resolution to support automated assessments of organisms ≥ 10 to <50 µm.

Figure 1. Image of a sample well from a SensoPlate Plus™ 384 plate, with 20 µL of artificial seawater added. An optical micrometer was placed below the well to allow resolution to be checked.

Figure 2 demonstrates additional suitability of these plates for analysis organisms ≥ 10 to <50 µm. Shown in the figure are the brightfield and epifluorescence images of the flagellate *Tetraselmis impellucida* (strain PLY 429; cultures were purchased from Reed Mariculture; Campbell, CA) in a well of a SensoPlate ™ 384, which is similar but slightly inferior to the SensoPlate Plus™ 384 wells as a result of its use of right angle versus tapered walls. The sample consisted of 20 µL of Instant Ocean® artificial seawater (Spectrum Brands; Atlanta, GA) with added *T. impellucida* (from a monoculture). The sample was stained with FDA and CMFDA according to the NRL protocol. Because *T. impellucida* are 6 to 12 µm in minimum dimension, they are at the small end of the ≥ 10 µm to < 50 µm size class. The images shown in Figure 2 demonstrate that organisms of this size can be identified in both brightfield and epifluorescence images.
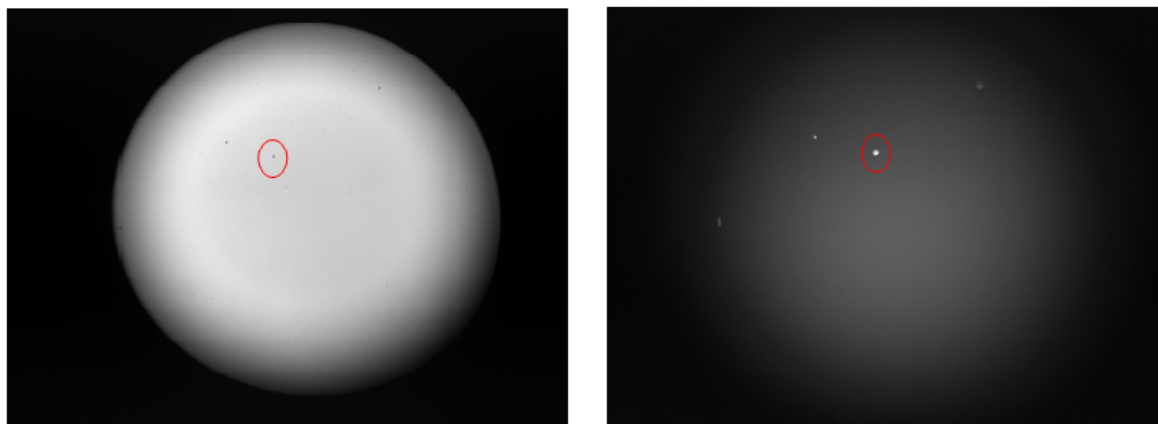
Figure 2. Brightfield (left) and epifluorescence (right) images of the flagellate *Tetraselmis impellucida*. in a well of a SensoPlate™ 384. The red circle indicates the position of the same *T. impellucida* cell in both brightfield and epifluorescence images.

Although initial results with these commercial sample well trays are promising and demonstrate the potential for their use in support of automated analyses, several issues complicate their use. First, even with a very small sample volume of 20 µL, the water column is sufficiently deep to allow organisms to swim into and out of the microscope's depth of focus. Second, the wells' edge effects make it difficult to visualize organisms at the perimeter of the well. These effects are obviated to some extent with SensoPlate Plus™ 384 well plates and their geometry. However, some distortion is still encountered with these wells because of microscope alignment issues that, despite assistance from Nikon and regular maintenance, have not been eliminated.

If image set data from sample wells were always of the quality shown in Figures 1 and 2, then this would be the preferred method for holding samples of organisms in the ≥ 10 µm to < 50 µm size class during the collection of image sets because it would use a COTS sample well tray. However, brightfield imagery generated from sample wells was often (but not always) affected by microscope misalignment that varied as the way the sample tray was held in place was adjusted slightly. This misalignment typically made the brightfield imagery appear spatially distorted, reducing the effective area over which organisms could be observed (i.e., smaller than the sample well itself). The epifluorescence image set data (which is the more important of the two modalities for analysis) was generally not affected by these microscope alignment issues. The data from this microscope modality, however, were affected by reflections, which created multiple signals when organisms were near well walls.

The four brightfield images in Figure 3 illustrate how the microscope misalignments resulted in spatial distortions in the brightfield image sets. Each image was collected from a different sample well containing a sample from field experiments conducted in March 2011. It should be noted that the magnification in Figure 3 is approximately twofold lower than in Figure 2. A review of these four images show that the upper left and lower right images suffer from minimal spatial distortions, while the images in the upper right and lower left are both spatially distorted (Fig. 3). The spatial distortion is indicated by the lack of roundness of the depicted sample well. Further, a review of these two images shows that portions of the sample well may actually be occluded as a result of the spatial distortions. This is indicated by the darkened region along the lower right portion of the perimeter of the sample well in the image on the upper right and as the darkened region on the right side of the perimeter of the sample well in the image on the lower left in Figure 3. It must be noted that, even with the potential for spatially distorted brightfield image data, automated counting and motility algorithms can still be applied successfully to the image sets that are

collected using COTS sample well trays. However, the ability to review archived image data (as well as the performance and robustness of the automated counting and motility algorithms) will be improved if brightfield image data can be collected without spatial distortions.

NRL examined an alternative method (unruled SR chambers) for holding samples. With this approach, the microscope magnification is adjusted to magnify an area on the SR counting chamber that corresponds to 20 µL volume. As described below, this approach provides a means to capture brightfield image sets without spatial distortions.
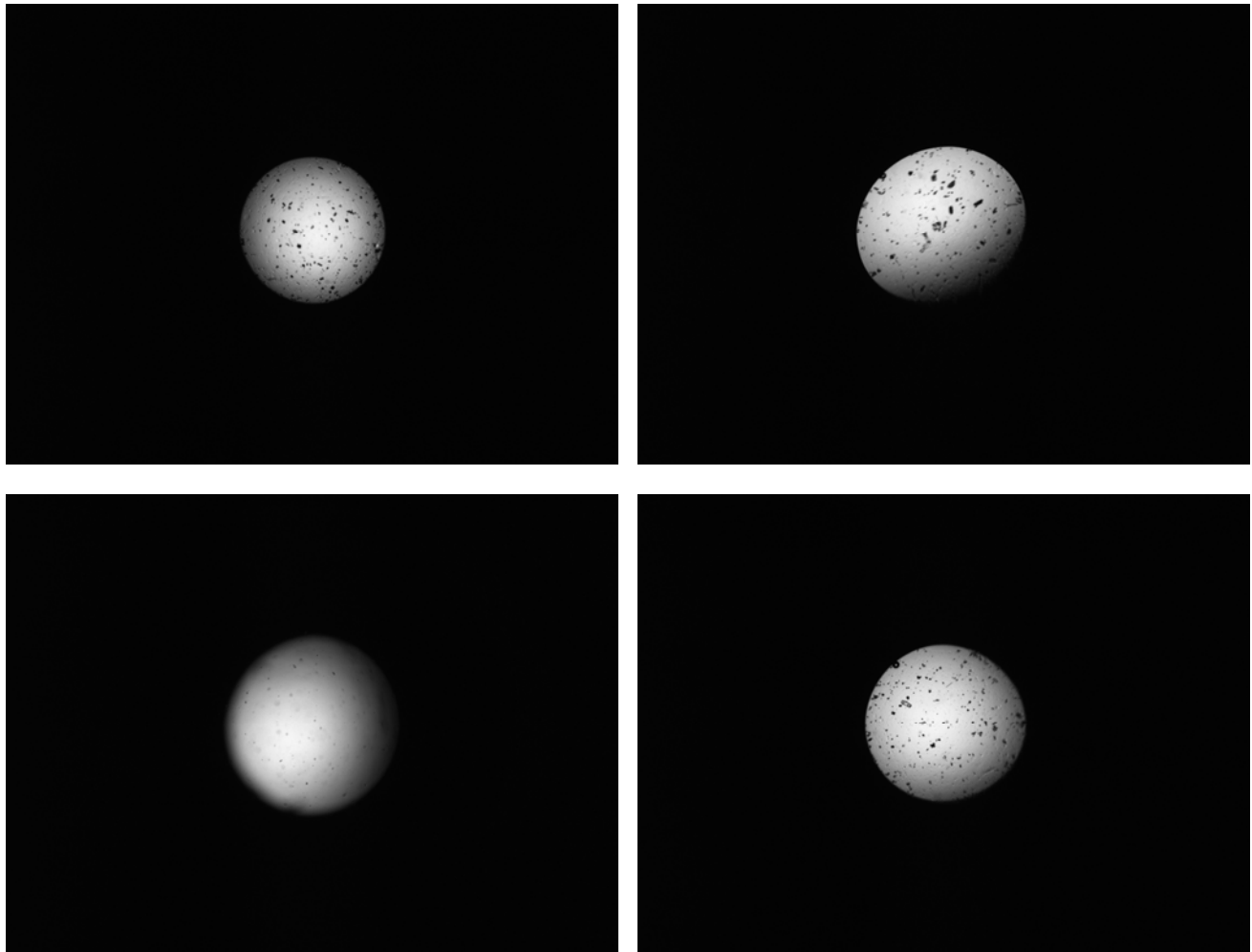

Figure 3. Brightfield images collected of samples using COTS sample well trays (SensoPlate ™ 384) with samples from a field experiment in March 2011. Images in the upper right and lower left are distorted; it is believed that microscope misalignments can result in occluded regions in the sample well. The other images are not distorted.

Figure 4 provides four brightfield images that were also generated from samples from the field experiments from March 2011. These images were collected using a standard unruled SR counting chamber with the microscope magnification set so the microscope's field of view encompasses a 20 µL sample volume. None of the images are spatially distorted using this method for holding samples under the microscope. This approach provides slightly lower spatial resolution than the sample wells (as the magnification is lower and the imaged area is larger in one dimension because of the camera's rectangular detector). However, the

spatial resolution is adequate for the automated analysis of samples with organisms in the ≥ 10 μm to < 50 μm size class. The major advantage of the SR approach is that the spatial information in the brightfield imagery is not distorted, which will make manual review of archived microscope image data easier to perform. Also, the greater degree of uniformity in the image data will also likely result in improved performance in the automation algorithm.
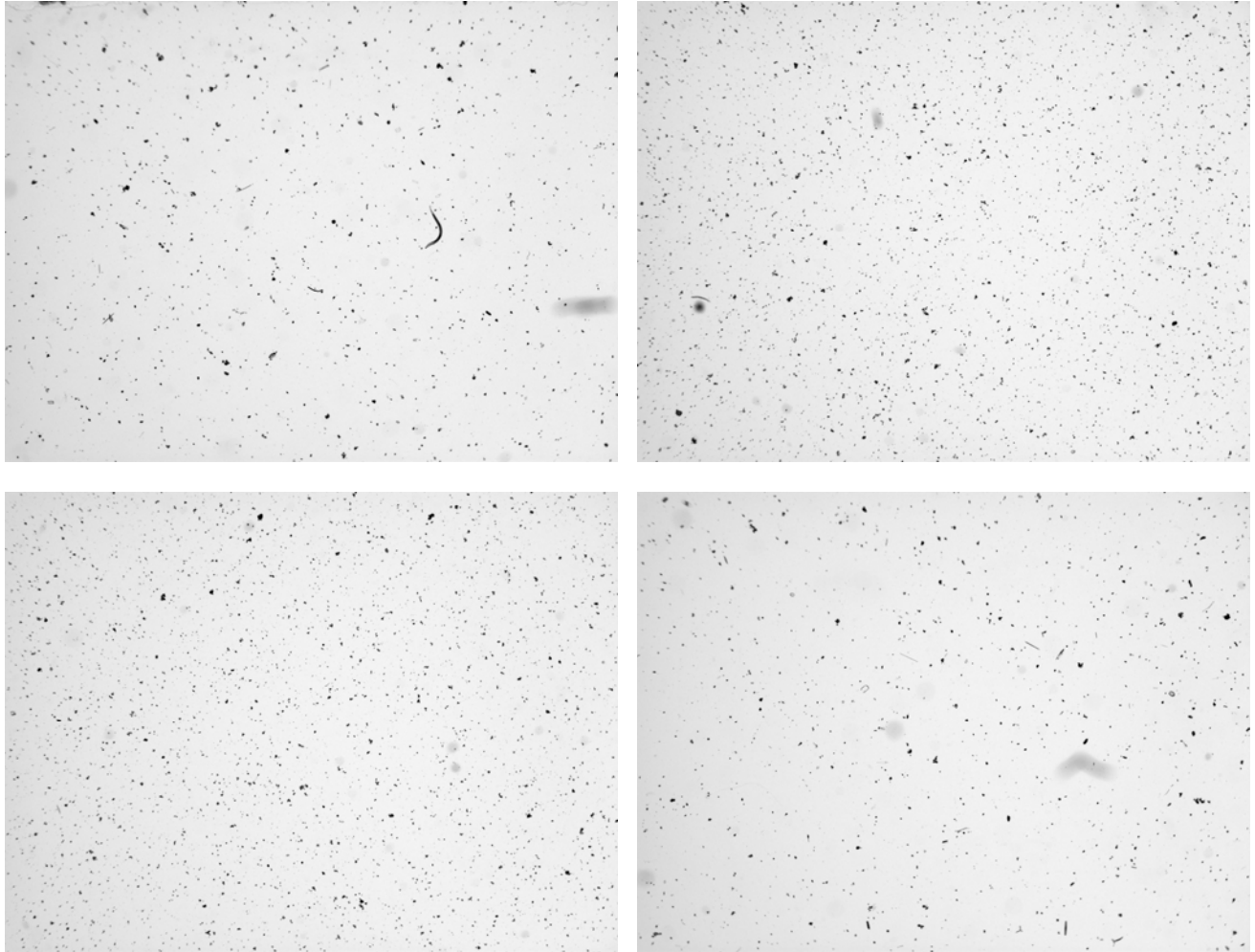

Figure 4. Brightfield images of samples collected using unruled simulated low volume Sedgwick Rafter counting chambers. The spatial distortions observed using COTS sample well trays were not observed in these image sets. Further, no regions in these images are occluded from view as a result of spatial distortions.

The work performed with both methods for holding samples (COTS sample well trays and SR) showed that issues with depth of focus and water column depth are the same for both of these approaches. In both cases, the water column depth is deeper than the microscope's depth of focus. The impact of water column depth, which can result in the spatial properties of the organism not being measurable and some organisms not being detected, is the subject of ongoing research.

NRL now is exclusively using unruled SR counting chambers to hold samples of organisms ≥ 10 μm to < 50 μm under the microscope. This decision was driven by the lack of spatial distortions in the brightfield image data and the lack of organism well edge reflections in the epifluorescent microscope modality. With

this approach, up to 50 individual 20 μL sample volumes could be interrogated on a single chamber (and each evaluated independently using the microscope's automated X-Y stage). This approach necessitates that organism be tracked and accounted for as they move into and out of the microscope's field of view.

3.2   Microscope

NRL worked with Nikon to design a microscope with the desired capabilities for use with a Q-Imaging 2000R Retiga IEEE 1394 camera system. The desired capabilities include:

- ➢ Ability to image a 3 to 5 mm diameter field of view with < 4 μm spatial resolution.
- ➢ Capability to collect images using brightfield illumination and epifluorescence
- ➢ Ability to rapidly switch between brightfield and epifluorescence modes under computer control
- ➢ Ability to move the micro-well tray or SR counting chamber under computer control so all micro-wells on a tray can be imaged
- ➢ Ability to focus the microscope under computer control
- ➢ Ability to monitor key system settings under computer control

3.2.1   NRL Microscope

NRL used the Nikon Multizoom AZ100 Multi-Purpose Zoom Microscope. As purchased, this microscope provides basic magnification of 5X, 10X, 20X and 50X with an 8:1 variable zoom available at each magnification. Implementation of this protocol does not require the four basic magnifications described above (Note the total magnification above is a combination of the objective magnification (variable) and the ocular magnification [10X]). To support the protocol provided in this report, the microscope is always operated at a basic magnification of 10X (1 X objective lens) with the variable zoom set to 3X (low volume SR counting chambers) or 4X (384 micro-well plates). Consequently, a single objective lens is used to support this protocol. This microscope was originally purchased with the Nikon AZ-FL Epifluorescence Attachment, which allows the microscope to operate in both brightfield and epifluorescence modes. The approximate cost of the microscope with the epifluorescence attachment (and all required peripherals) was $30,000.

3.2.2   Light Sources

In December 2010, Nikon integrated a new epifluorescence light source into the imaging system (Lumen Pro™ Fluorescence Illumination System [Prior Scientific, Rockland, MA] equipped with a 200 W metal arc lamp). This additional light source allowed faster and more reliable switching between brightfield and epifluorescence light sources through the software interface. The cost of these light sources and the appropriate control software was approximately $10,000. This light source replaced a computer controlled light source installed by Nikon in July 2009. Work performed at NRL indicated that the first computer controlled light source did not provide the reliability and timing precision required for this application.

3.2.3   Sample Stage

In October 2009, Nikon integrated a computer controllable, motorized X-Y-Z stage to the NRL microscope. This stage allows each sample well on the sample well plates to be moved into position (and image sets collected) under computer control. The stage also provides the capability to focus the microscope under computer control. The cost of this motorized stage was approximately $15,000.

3.3   Imaging and Image Acquisition Software

Nikon NIS-Elements Advanced Research Imaging System Software (Elements) is used to control the microscope system during automated data collection. This software controls the entire image acquisition process, the storing of image data into a single *.nd2 file, the switching of the light sources during image acquisition, and the movement of the motorized stage when multiple sample wells are evaluated in sequence on a single multi-well plate. The software also controls the camera settings and provides image display and image processing capabilities. The cost of this software package was approximately $ 3,600.

3.4   Camera

NRL uses a Q-Imaging 2000 R Retiga IEEE 1394 camera, which provides 1600 x 1200 pixel resolution using a large area charged coupled device (CCD) detector that provides high light sensitivity (compared with consumer CCD-based imaging systems, such as video cameras). The camera is fully controllable using the NIS-Elements software. For this application, the monochrome, uncooled version of the camera is recommended. The cost of this camera is approximately $7,000. An even higher resolution camera (that will result in still more improved spatial resolution) that uses a 2048 x 2048 pixel detector is available at a cost of approximately $9,000. Both cameras are fully compatible with the Nikon Elements Software.

3.5   Computer

To complete the system, a PC with at least two RS-232 ports, four USB-2 ports, and an IEEE 1394 (Firewire) interface is required. The cost of an appropriate PC and display for the imaging system is estimated at $3,000.

3.6   Total Costs

The total cost of a new system for implementing the protocols described in the next section of this report is approximately $69,000 (Table 1). With the upgrades made in its microscope system, the imaging system at NRL is now also configured to implement the protocols provided in the next section of this report.

Table 1. Equipment summary and costs.

Item	Model	Approximate Cost ($)
Microscope	Nikon Multizoom AZ100 Multi-Purpose Zoom	30,000
Light Source	Nikon Computer-Controlled Light Switcher	10,000
Sample Stage	Nikon Motorized X-Y-Z Stage	15,000
Imaging Software	Nikon NIS-Elements Advanced Research Imaging System Software	3,600
Camera	Q-Imaging 2000 Retiga IEEE 1394 camera	7,000
Computer	PC with 2 RS-232, 4 USB-2 Ports & IEEE 1394 (Firewire)	3,000
Analytical Software	Government developed	0
TOTAL		**~$69,000**

3.7   Settings

Following the October 2009 and December 2010 updates to the microscope system, the majority of system settings are either directly monitored by the computer or entered by the microscope operator into the spreadsheet (as discussed in the next section). Many of the parameters entered into the spreadsheet can be corroborated, as these same data are recorded in the *.nd2 files. There are currently four parameters that are adjusted manually by the microscope operator that cannot be corroborated in the *.nd2 files. First, the operator manually adjusts (or ensures) that the variable zoom level is set appropriately to either 3X (low volume Sedgwick Rafter) or 4X (384 Multi-well plate) for implementation of the data collection protocol. The operator is asked by the spreadsheet to confirm this zoom level prior to the start of data acquisition. Second, the filter cube position is manually set to position 2 for the Green Fluorescent Protein (GFP) filter cube. Again, the operator is asked by the spreadsheet to confirm the filter cube position prior to the start of data acquisition. Third, the microscope operator needs to ensure that the objective best matched to the sample well or imaged area (20 µL volume of a standard 1 mL unruled SR counting chamber is used). The operator is also asked to corroborate this point prior to data acquisition. Lastly, the microscope operator needs to ensure that the neutral density (ND) filters are in the proper configuration: the ND2 filter should be not engaged and the ND8 and ND16 filters should be engaged. The microscope operator is asked to corroborate this configuration prior to data collection.

3.8   Data Analysis Software

A series of algorithms was developed by NRL to analyze the images collected and stored by the Elements software. The algorithms analyze the images in the selected file and store the results in the file's associated spreadsheet file. This government-developed software for analyzing the collected data will be made available at no cost.

4   DATA MANAGEMENT AND DATA COLLECTION PROTOCOLS

Two protocols have been developed by NRL to satisfy the requirements of standardized data collection: the Data Management Protocol and the Protist (i.e., organisms ≥ 10 µm to < 50 µm) Data Collection Protocol. They are intended to be used in concert, and they ensure that system settings are properly set, documented, and recorded. As described below, in many cases, redundant information is recorded in the two major outputs: an Excel Spreadsheet, which contains comments by the operator and lists the settings used, and an *.nd2 file, which contains the diascopic (DIA, transmitted light [brightfield illumination]) and epifluorescence (EPI) image sets. NRL has successfully used both protocols to support its data collections since August 2009.

Implementing the protocols is a three-step process. First, the operator sets up the directories and generates a test specific Excel spreadsheet. This process is described in Sections 4.1 and 4.3 (step-by-step instructions are detailed in Appendix A). Next, the operator next sets up the microscope and collect the image data. This is described in Section 4.2 and 4.3 (and in Appendix A). The operator then runs the analysis routines on the collected image set data, which is described in Section 5.

4.1 Data Management Protocol

The Data Management Protocol serves two purposes. First, it provides a standardized means of storing data with a file naming convention into a directory structure that allows specific-test data to be easily identified. Second, it produces a test-specific spreadsheet that documents key system settings and allows the microscope operator to enter test-specific comments following a review of the image set after it is collected.

4.2 Data Collection Protocol for Organisms ≥ 10 to <50 µm

The purpose of the Protist (i.e., organisms ≥ 10 to <50 µm) Data Collection Protocol is to ensure that standardized image data sets are collected. It is used to set the microscope and camera settings prior to data collection and to collect the standardized image sets in an *.nd2 file format following system setup. At this time, only two camera-setting parameters are specified by the microscope operator for the GFP (green fluorescent protein) optical configuration used for epifluorescent illumination: the camera exposure time and the camera gain settings. Work continues in the refinement of the optimum camera settings for GFP measurements, with the majority of recent data being collected with a camera exposure value between 70 and 100 milliseconds (ms) and a gain factor of 1.0. The total magnification used is between 30X and 40X (1 X objective*10X ocular *3 or 4X zoom) depending on the sample well or SR chamber configuration. Many other parameters, such as lamp brightness, are automatically set by configuration files (not editable) accessed by Nikon Elements and are not user-selectable. These metadata are stored in the *.nd2 file.

4.3 Using the Protocols – Summary Steps

Brief descriptions for using the Data Management Protocol and Protists Data Collection Protocol follow. The complete protocols are provided in Appendix A. The protocols assume that the microscope operator has basic familiarity with the AZ100 microscope and the Nikon Elements Software.

Prior to running the data management and collection protocols, the sample should have been concentrated and stained. A 20 µL subsample should have been placed in the sample well and the sample plate placed onto the sample stage. Note that the stained samples should be analyzed within 20 minutes.

There is ongoing work to modify the Excel spreadsheet file used in support of this protocol. These modifications are the result of the extensive amount of testing that has been performed in 2010 and 2011. As this is an ongoing process, the Excel spreadsheet that was described in last year's Protocol for Automated Protist Analysis is also described in this document. When the new spreadsheet file is finalized, it will be described in detail in next revision of the protocol.

4.3.1 Data Management – File naming

The observer starts by creating a new experiment folder for each new *.nd2 file. This folder will contain all images taken for each sample well analyzed during the experiment. A strict file and folder naming convention allows all replicate or subsample data to be associated with the corresponding Excel spreadsheet. The folder should be labeled with the date and a sequential number. For example, a folder Desktop > ANS > Year > Month > Day > Treatment > Run Folder could be Desktop > ANS > 2011 > 03_Mar > 14 > tetraselmis_100ms > 20110314_tetraselmis_100ms_001. The file itself should be named to match the run folder - (20110314_tetraselmis_100ms_001).

Once the folder name is established, an Excel data sheet template (.xls or .xlsx) is saved to the folder with the Excel file having the same name as the folder. If necessary, the operator may need to change the Excel security setting to medium to allow Excel to open embedded macros. The operator then opens the Excel worksheet and enters appropriate data for the run. All entries, including redundant data, must be filled before saving. Comments can be added to the worksheet after the .nd2 images are reviewed.

4.3.2   Data Collection

With the camera and both light sources connected to the computer, the Elements software is turned on. After the microscope is focused and the optical pathway sent to the camera, the operator opens a live preview in Elements. Clicking the DIA optical configuration on the toolbar allows the operator to focus the camera based on the live preview window. The operator then checks and records the physical microscope settings. The operator then changes to the GFP optical configuration on the toolbar and verifies the GFP settings.

Focusing (eye or camera) is not trivial as a result of the depth of the water column. This is harder to accomplish accurately for organisms ≥ 10 µm to < 50 µm as compared to organisms ≥ 50 µm, however with experience skilled and experienced microscope operators should be able to accomplish this function. It is important to note that we are recommending a paradigm shift in the way the microscope is operated: the focus is on the complete well, not on individual organisms. Focus becomes more important here, as magnification and the ultimate resolution of the individual organisms is obtained by zooming in on the images rather than on the organisms.

With all settings verified and recorded, the operator selects which folder to save the images in and enters the first filename as "filename_001". The Elements software will automatically increment the number at the end of each run. The operator sets the interval to 1 sec and the duration to 31 sec. (Note that the first image from each microscope modality is deleted to eliminate problems that frequently occur with these two images during data collection). The number of loops is automatically calculated (for 31 sec, there will be 32 loops because the first loop starts at time zero). A small flag icon should be visible in the loops tab (see Appendix A, Fig. A-15). This indicates that the routine will be forced to finish when the routine reaches the specified number of loops. If this icon is set on the duration tab, then the routine will be forced to quit at exactly 31 sec). Finally, the operator checks the Lambda tab (which indicates the illuminations source) to verify that the first Lambda is DIA and the second is GFP.

The operator then clicks "Run now" to start automatically collecting images of the sample under the ocular. As indicated earlier, this only results in the collection of the image sets. Analysis of the image sets occurs separately after the completion of the image acquisition.

5   DATA ANALYSIS AND BASIC ALGORITHM DESCRIPTION

The Data Analysis algorithms are executed using an analysis program as a stand-alone, Windows-based application. (This analysis software will be available at no cost.) When run, the application asks the operator to specify a *.nd2 file to be analyzed. The *.nd2 file associated with a given test is first parsed into brightfield (DIA) and epifluorescence (GFP) image sets. The automated counting algorithms operate on both of these parsed image sets. For organisms ≥ 10 µm to < 50 µm, there are two analyses performed. First, the number of organisms that have produced measurable epifluorescence signals are counted for each image in the GFP image set as well as across the complete GFP image set. For organisms in the ≥ 10 µm to

< 50 μm size class, the detection of an organism with a measurable epifluorescence signal provides a strong indication that an observed organism is viable.

Following the initial counting of organisms with measureable epifluorescence signals, motility algorithms are applied to the DIA and GFP image sets. The motility algorithms provide a display of the DIA and GFP image sets as movies and tabulate the results from individual image pair analyses (that are conducted to identify motile organisms in both the DIA and GFP image sets) as well as the results obtained across each image set and across the microscope modalities. The motility algorithms produce the number of organisms that have moved during the observation window.

The results of the two counting algorithms are next combined, by performing a spatial correlation of the cells identified by each of these algorithms. The combined result is provided as a screen output, which provides the number of living organisms detected in the analyzed data. Additionally, the data analysis routine outputs its final (as well as intermediate) counting results directly into the spreadsheet described in the Data Management Protocol.

Results are written to the Excel spreadsheet associated with the given *.nd2 file analyzed. The overall result (the number of viable organism per mL), which is based on the analysis of the entire image set is written to the spreadsheet. The software additionally writes out interim motility results such as those generated on an image pair basis for the both the DIA and GFP image sets as well as the individual image results obtained from counting the number of organisms with measureable epifluorescence signals in the GFP image sets. Lastly, image data are available for review by the operator. This allows the operator to corroborate automatically generated results and, if required, to amend results based upon their observations of the image set data.

It should be noted that final data interpretation rests with the skilled researcher. Results from recent studies at NRL indicate that motion of organisms is a key factor in determining live/dead status, even for organisms in the ≥ 10 μm to <50 μm size class. Varying the threshold when analyzing/reviewing archived images can improve the ability to detect motile organisms that are only weakly stained or ones with low contrast in brightfield images. An in-depth discussion of recent research conducted with complex natural samples can be found in "Automated Protist Analysis of Complex Samples: Recent Investigations Using Motion and Thresholding".

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APPENDIX A.   PROTOCOLS FOR AUTOMATED PROTIST ANALYSIS

The protocols on the following pages provide step-by-step instructions for creating data files, capturing images of treated samples, and analyzing the collected images. The protocols and accompanying screen images were developed using the equipment described in the preceding text. As mentioned in the main body of this document, the specific spreadsheets that are used in support of this protocol are currently being modified to make data collection easier and more efficient. These changes will be documented and included in the next revision of the Protocol for Automated Protist Analysis. The government-developed software for analyzing the collected data is in the public domain and is available from NRL.

A.1   Data Management Protocol File Naming and Convention

Create a new experiment folder for every new .nd2 file.

Desktop > ANS > Year> Month > Day > Treatment > Run Folder

The Experiment Folder should be labeled with the date and then a chronological number. Examples:

Desktop > ANS > 2011 > 03_Mar> 14 > Protist_SR > 20110314_Protists_SR_001 (e.g., replicate or subsample 001)
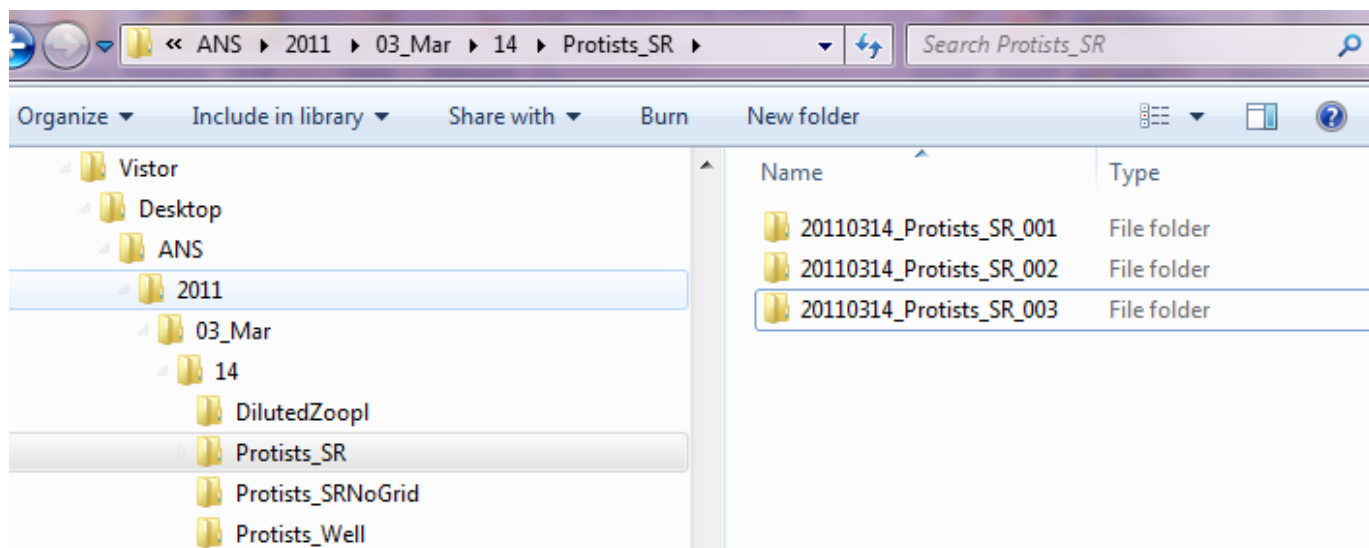
Figure A-1. File naming and convention.

The file itself should be named to match the Run Folder: 20110314_Protists_SR_001.nd2 (Figure A-1).

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A.2 Data Management Protocol Excel Data Sheet

Each Treatment Folder should have a copy of the AZ100 Excel data sheet.

1. The template will be located in the ANS folder. Save your edited version in the Treatment folder with a filename identical to the **sample description** (e.g., 20110314_Protists_SR.xlsx; See Figure A-2).
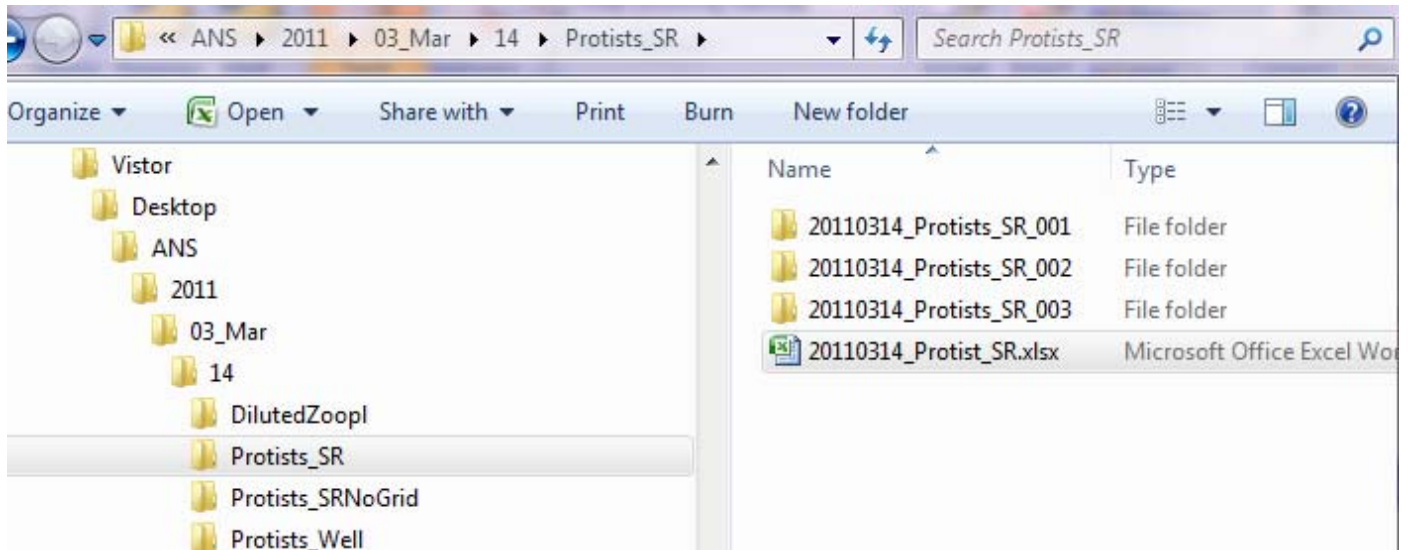
Figure A-2. Excel data sheet in Treatment folder.

2. You may have to configure Excel to be able to open the macro called out in the programs. If prompted, enable macros to run in the file.

In Excel 2003 running in XP, go to the Tools menu > Macro > Security (Figure A-3, top panel).

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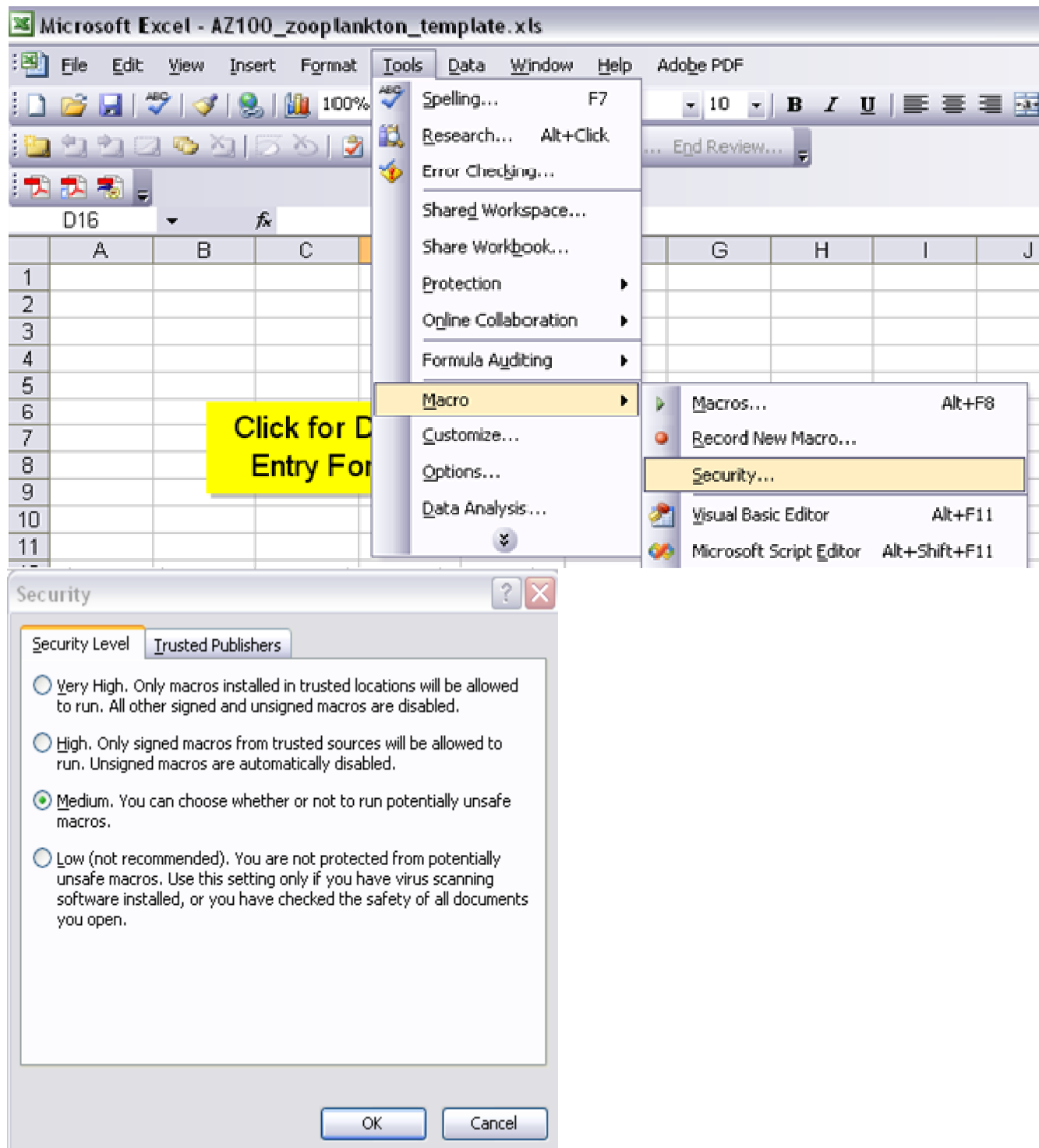
Figure A-3. Excel data sheet security menu (top panel) and security selection level (bottom panel).

Choose Medium security (Figure A-3, bottom panel), click OK, close Excel, and reopen it.

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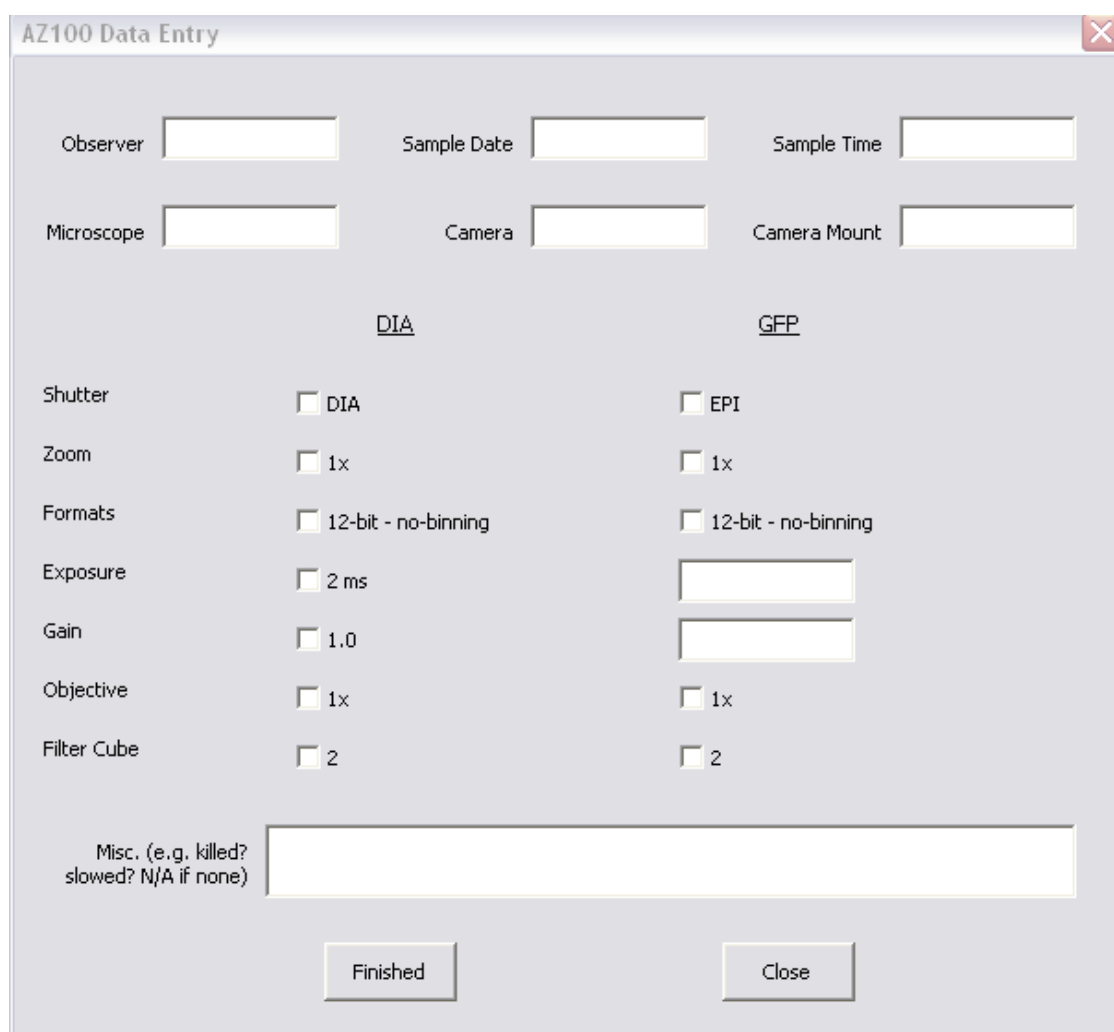
3. On the Data Entry worksheet, click on the yellow Data Entry button (Figure A-4).

Figure A-4. Data entry form button.

Entries or checks are required for all data fields (otherwise, the file cannot be saved; See Figure A-5).

Figure A-5. Required data fields in Excel data sheet. Note that the data entry form will be updated in the final version.

4. After each .nd2 file is recorded, view the images and write observations in the appropriate 'Comments' worksheet in the Excel file (Figure A-6).
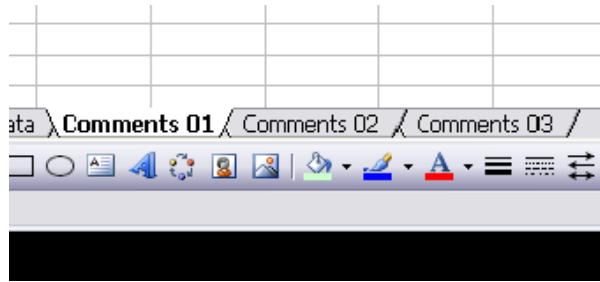
Figure A-6. Excel data sheet comment pages for each .nd2 file. Note that the operator comment section will be modified and updated in the final protocol.

A.3   DATA COLLECTION PROTOCOL

A.3.1   Initial Set-up

1) Before opening Elements program, turn on the camera and both lamps.

2) Place your sample on the stage and focus the microscope using the oculars. Once the sample is in focus, pull out the knob to change the optical pathway from the oculars to the camera.

3) In Elements, open up a Live Preview window. You can do this by going to the Acquire menu (Live – Fast), by clicking the "Play" Icon on the toolbar (see Figure A-7), or by pressing the '+' key on the keyboard.
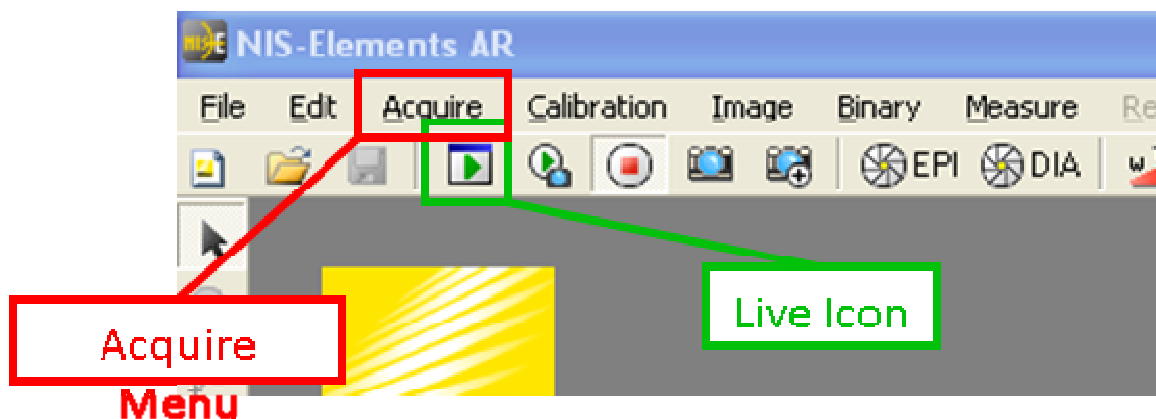
Figure A-7. Acquire menu and Live icon.

A.3.2   Camera Set-up

4) Click on the DIA optical configuration in the toolbar, *not* the control button (Figure A-8).

The optical configuration buttons are preset with settings for the camera exposure, image gain, and objective lens and filter cube, etc.

These settings will be used when you take a time series of images.
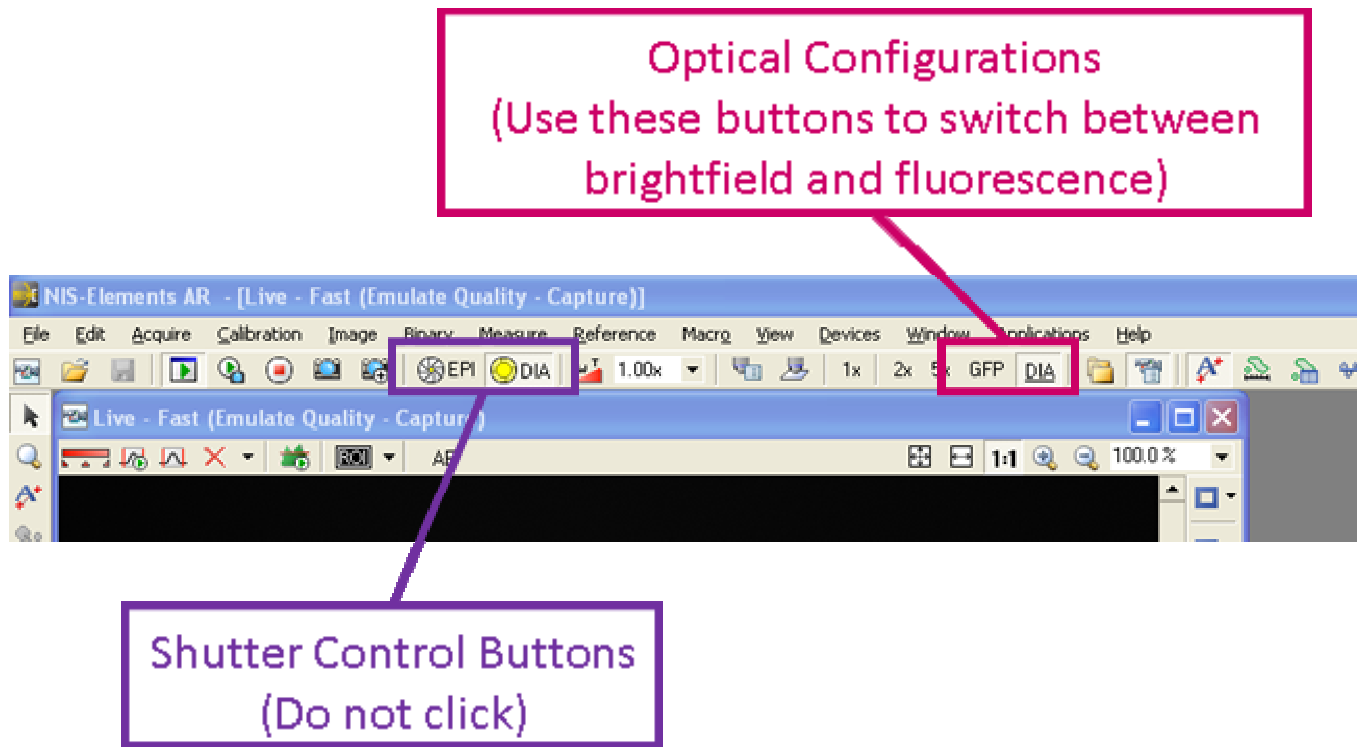
Figure A-8. Optical configuration and shutter control buttons.

A.3.3   Camera Focus and Settings

5) Now use the microscope focus to adjust the image in the Live Preview window. You may need to zoom in on the preview to make sure it is as crisp as possible. (Figure A-9).
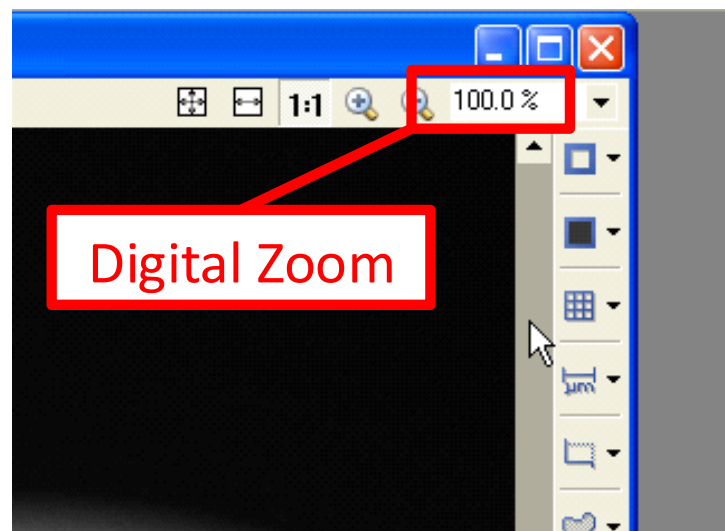

Figure A-9. Digital zoom button.

6) Check the physical microscope settings:

- The GFP Band Pass filter cube is in place (2)
- The 1.0X objective is in place
- Zoom is at 3.0X for SR counting chambers or 4.0X for 384 microwell plates
- At the bottom right of the base, the ND16 and ND8 filter knobs are pulled out (keep the ND2 knob pushed in; ND = neutral density)

A.3.4 Verification of Elements Settings - DIA

7) Check all your Elements settings. With the DIA optical configuration button (orange box, Figure A-10) selected, there are several things to look for (Figure A-11):

   i.   Microscope camera status is set to live.

   ii.   Zoom is set to 3X or 4X, as appropriate to the specific sample holder

   iii. The formats for live and for capture are both set at 12-bit - no binning

   iv.   The exposure time is 5 ms

   v.   The hardware gain is 1.0

   vi.   On the manual microscope pad menu, the 1X nosepiece is selected

   vii. The DIA filter turret (the first gray icon) is selected

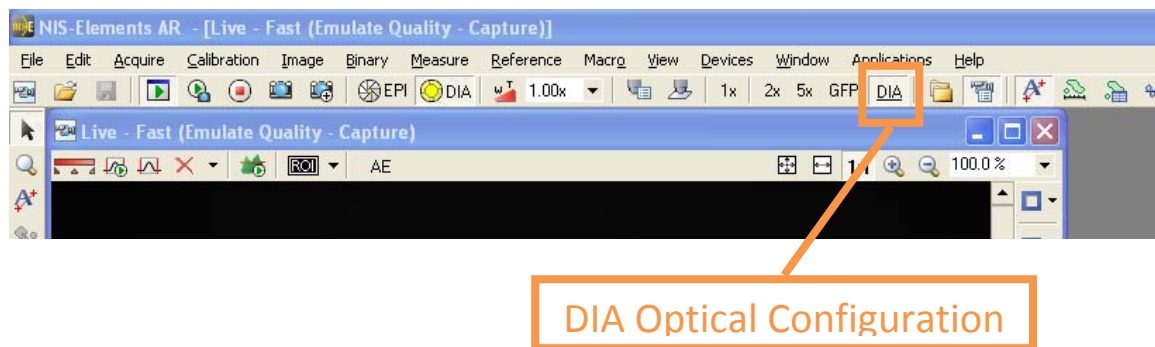(Note that some of these settings may change over time and depending on the sample.)

Figure A-10. DIA optical configuration button.

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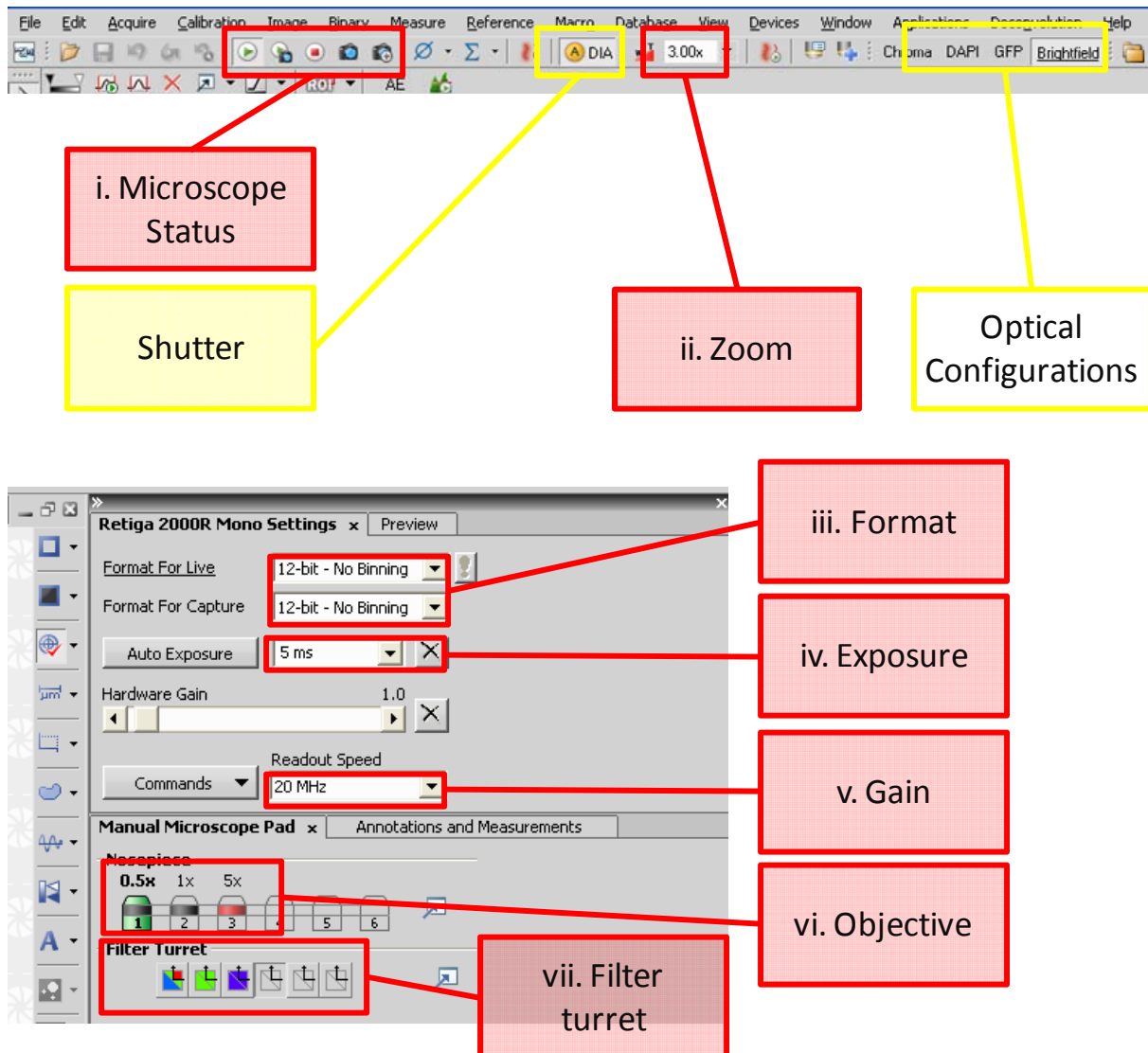**Research & Development Center**

Figure A-11. DIA setting summary.

A.3.5 Verification of Elements Settings - GFP

8) Now click on the GFP Optical Configuration button (green box, Figure A-12) and double check the GFP settings:

- The EPI shutter button is selected
- The variable zoom is set to 3X or 4X, as appropriate to the specific sample holder
- The formats for live and for capture are both set at 12-bit - no binning
- The exposure time is 100 ms (this is a variable now)
- The hardware gain is 1.0 (this is a variable now)
- On the manual microscope menu, the 1X nosepiece is selected
- The GFP filter turret is selected

(Note that some of these settings may change over time and depending on the sample.)
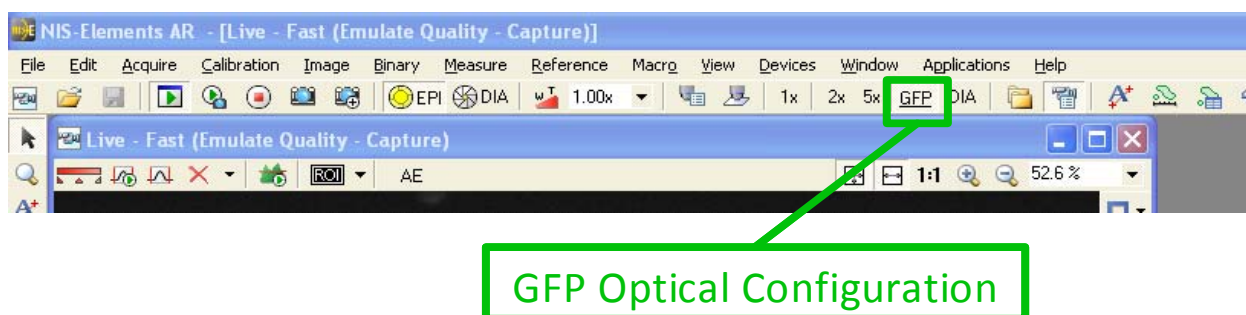

Figure A-12. GFP optical configuration.

If you make changes to the exposure or gain while an Optical Configuration is selected, the new values will be propagated as part of the preset values. That is why you *must* ensure the settings are correct before beginning data collection.

A.3.6 Manual Changes

You can reset the presets by manually changing the above settings, going to the Calibration menu at the top of the screen, and choosing Optical Configurations. Make sure the correct optical configuration is selected in the menu to the left and then click on "Assign Current Camera Settings" or "Assign Current Microscope Settings" (Figure A-13).
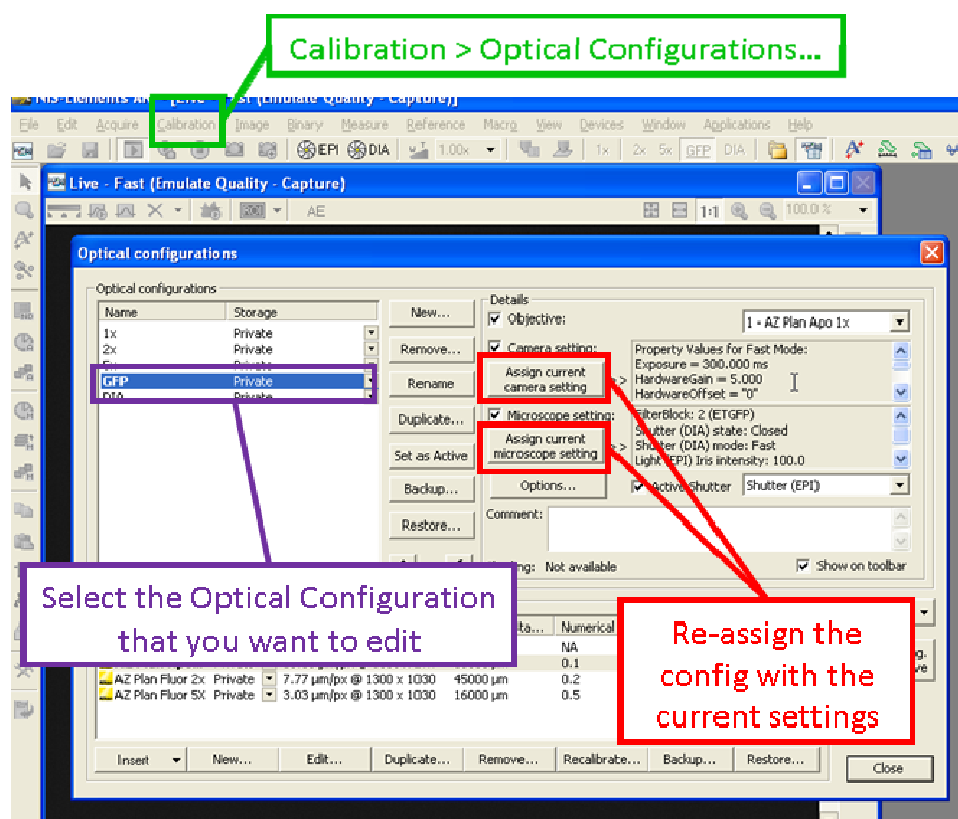

Figure A-13. Assign current settings.

A.3.7 Final Step - Designating Acquisition File

9) At this point, you have checked all your settings and the camera is in focus. Go to the Applications menu and choose Define/Run Experiment (Figure A-14).
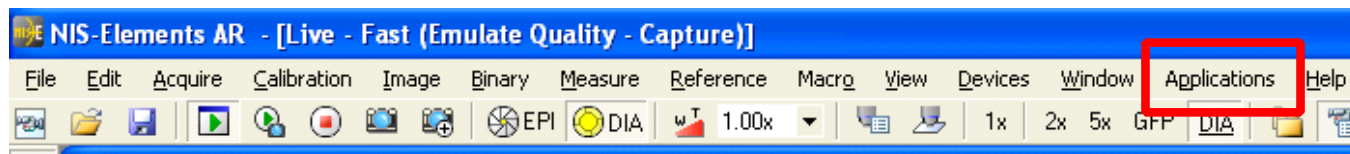

Figure A-14. Applications button.

10) Click on Browse to choose the directory to which to save files. Enter your file name followed by 001 (Elements will automatically increase the number at the end of each run). Set the Interval to 1 sec and the Duration to 31 sec. The Loops will automatically change to 32. Assure that the flag icon appears in the Loops tab. If the icon is in the Duration tab, click on the Loops tab to move the flag icon (Figure A-15).
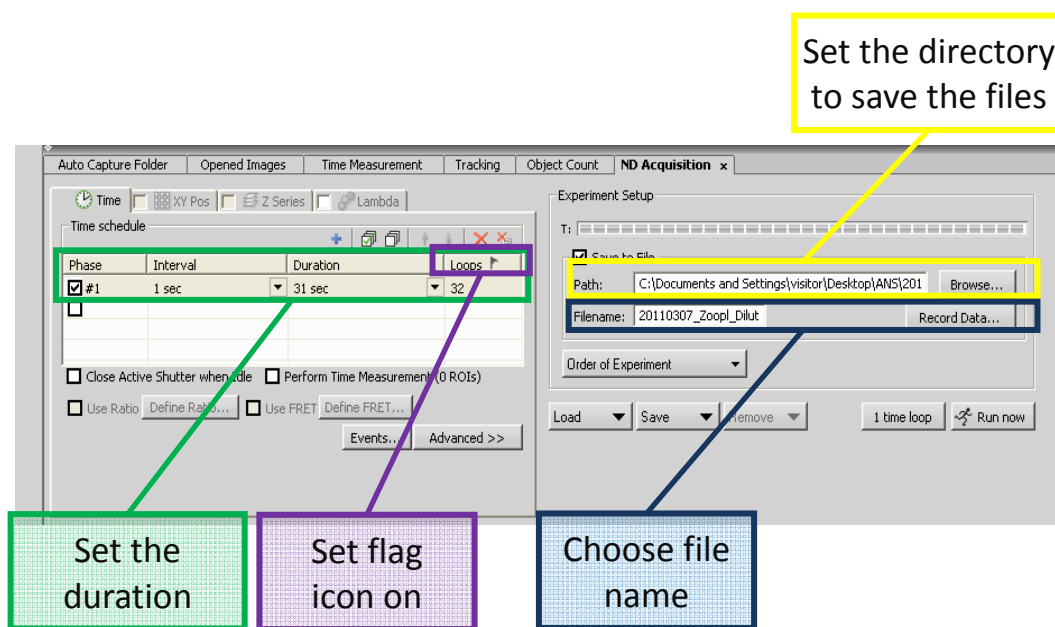

Figure A-15. File designation set-up.

A.3.8   Acquire Data

11) Click on the Lambda tab (Figure A-16). Make sure the first Lambda is DIA and the second Lambda is GFP.
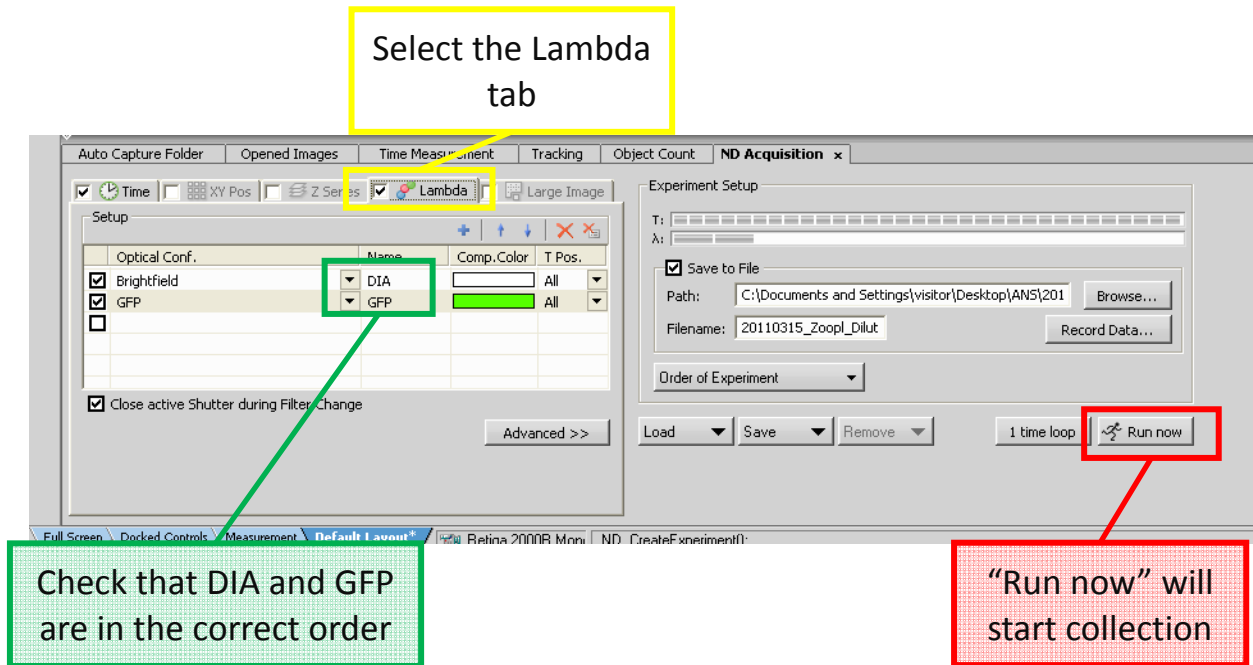

Figure A-16. Setting light source sequence.

12) Everything is set up, so click "Run now" to start the time series (Figure A-16).

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