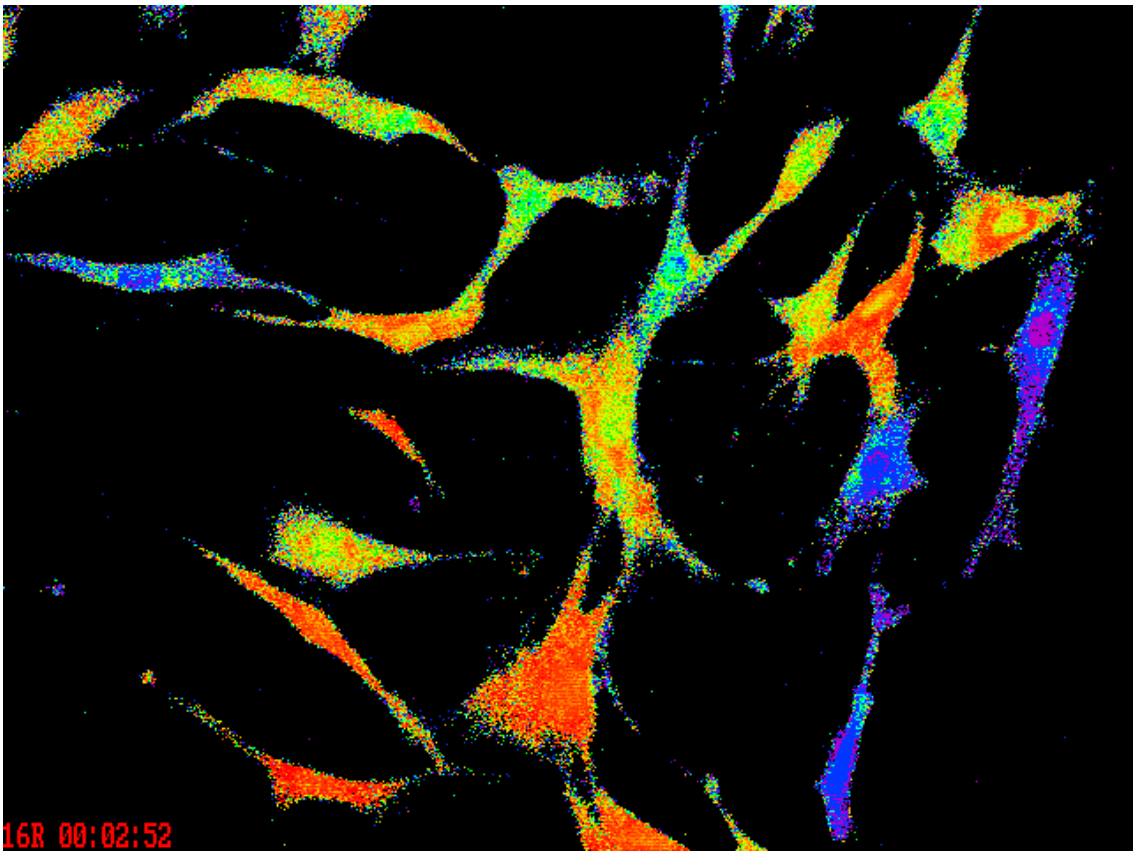
InCyt Im2™
Dual-Wavelength Fluorescence Imaging System



USER'S MANUAL
For Windows 10 & Sutter Wheel Excitation

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I. INTRODUCTION

"To promote scientific progress by providing researchers high-performance instrumentation that is easy to use at an economical price"

This simple statement is the driving force behind Intracellular Imaging Inc. (I^3). Our goal is to provide your lab with systems that will be used on a regular basis to obtain data that will move your research forward.

We are confident that you will be pleased with your *InCyt Im2*™ imaging system. It will prove to be a real *"workhorse"* instrument -- easy for everyone in your lab to learn and use, so that you can produce the maximum amount of data in the least amount of time.

Your system has been thoroughly tested before it leaves Intracellular Imaging. Our representative will set up the system in your lab and conduct a complete "hands-on" training session with live cells.

This manual will serve as a guide for new users and as a refresher for experienced users. If you have a question not covered in this manual, please to not hesitate to give us a call.

What you should know before you use the system

This manual was written with the assumption that the user has a working knowledge of the following:

1. Microsoft Windows 10. To familiarize yourself on the use of programs in the Windows 10 environment, please consult the Microsoft User's Guide. An on-line version of the guide can be found by pressing the **<START>** button in the lower left of your desktop screen and going to **<Get Help>**.

2. The biology of intracellular ion channels and ion mechanics. For details on the theory of this subject, please consult related publications and references such as:
   Mason, W.T., "Fluorescent and Luminescent Probes for Biological Activity" Academic Press, 1999.
   Nuccitelli, R., "A Practical Guide to the Study of Calcium in Living Cells" John Wiley & Sons, 1982.
   You can also check our Internet Home Page, www.intracellular.com for some references to other industry information sources. Your *InCyt Im2*™ system can be used with most of the hundreds of fluorescent dyes now available as cellular probes. This manual is written with a focus on calcium (Ca^{2+}) measurement using the dye Fura-2.

3. Fluorescence Microscopy and the use of a fluorescence microscope.

II. SYSTEM PARTS CHECK LIST AND SET-UP

A. Parts

Your Turnkey *InCyt Im2*™ System was shipped with the following parts:

- Imaging Workstation & Monitor
- Pre-Loaded Software:

  InCyt Im2™ Basic Image Acquisition and Analysis Software (dual-wavelength)

  InCyt Im1™ Basic Image Acquisition and Analysis Software (single-wavelength)
- Software Security Dongle (USB format)
- Software Backup USB Drive
- Mouse Pad
- Surge Suppresser/Power Strip
- Allen Wrench Set
- UV Goggles
- Standard Dish (for holding up to 6 calibration standards)
- Microscope (if ordered) with camera relay lens
- Fluorescence light path for existing microscope, consisting of the manufacturer's epi-fluorescence optics with Liquid Light Guide (LLG) collimator, an Intracellular Imaging Groony™ adapter, or a 3rd-party LLG adapter, unless your microscope was already LLG-ready.
- Filter cube fitted with dichroic cube and emission filter suitable for fura-2, GFP, and BCECF
- Liquid Light Guide
- Basler ac720-520um Low Light Level CCD Camera; 12-bit CMOS
- Camera control cable
- 175 watt xenon arc illuminator
- Sutter Lambda 10-B Controller and 10-Position Filter Wheel, with 340 & 380 excitation filters installed
- User's Manual

IF YOU ARE MISSING ANY ITEMS, PLEASE CONTACT YOUR INTRACELLUAR IMAGING OR YOUR DISTRIBUTOR IMMEDIATELY!

NOTE: We recommend that you keep as much of the packing material as possible, just in case any item must be returned to Intracellular Imaging for repair. Of particular importance are packaging for the microscope and computer.

II. SYSTEM PARTS CHECK LIST AND SET-UP

B. Setup

Step 1 -- Unpacking the Workstation

Unpack the box with the computer Workstation. Decide where you will place the Workstation, on the bench or on the floor. However, be aware that the system Control Box and the back of the camera must be within the reach of the Camera Control Cable, which attaches to the back of the Workstation. Turn the Workstation so you can easily reach the back connections.

You should not have to use any of the software disks or USB drives in this box, because all necessary software is preloaded on the Workstation. However, keep this software in a safe place, because it will be critical to recovering quickly from any CPU or software failure.

Be sure to remove all other items from the Workstation box.

Step 2 -- Making the Workstation Connections

A connection diagram is provided in an insert in your manual. The exact position of the Workstation connections will vary from system to system.

Connect the keyboard and mouse. Their plugs are color coded so the connections are easy to find.

Attach the Camera Control Cable and Software Security Dongle to the color coded USB ports on the computer.

Plug the power cord into the Workstation.

Step 3 -- Setting up the Monitor

Unpack the Monitor. This is the only item in this box. Connect the Monitor cable to the Workstation using the HDMI port on the computer (or to a color coded modified USB port using a combination USB-HDMI cable).

Plug the power cord into the Monitor.

Step 4 -- Power Connections

Intracellular Imaging has provided a Surge Suppressor for use with your system. Plug all components EXCEPT the Xenon illuminator into the Surge Suppressor. The 175-watt Xenon Illuminator MUST be plugged into the wall or a separate surge suppresser. **The 175-watt Xenon Illuminator must be turned on when everything else is off**. The Illuminator firing requires a short burst of 23,000 volts and causes a great deal of RF interference, which can damage components that are close by and operating.

II. SYSTEM PARTS CHECK LIST AND SET-UP

Step 5 -- Setting Up the Microscope

See microscope instructions. The epi-fluorescence optics and dichroic filter cube will be already installed in your microscope.

Step 6 -- Setting Up the Illumination System

The illumination system consists of the Xenon arc lamp Illuminator, wavelength changer, and the Liquid Light Guide. For the Standard system the wavelength changer will consist of a Sutter filter wheel and a control box for the wheel. The filter wheel may be mounted inside the Xenon lamp housing or externally, depending on the arc lamp configuration. Unpack these items.

Decide where you want to place the Microscope and the Monitor.

Set-up the arc lamp Illuminator with the black output port facing out. Make sure the unit's switch is off. Plug the unit into the wall or separate surge suppresser. Make sure there is space around the Illuminator for ventilation.

For Filter Wheels external to the arc lamp: Place the Filter Wheel onto its stand (see Sutter manual for instructions) so that the base extends toward the side of the Filter Wheel with the motor. Adjust the height of the Filter Wheel on the stand so that the wheel coupler is at the same height as the output port on the arc lamp. Slide the coupler over lamp port, and secure it with the thumbscrew. Tighten the wing nuts on the Filter Wheel to lock its position on the stand.

If it is not already installed, screw the Light Guide/Focusing Coupler into the open port of the Filter Wheel facing away from the Lamp. Remove the light guide adapter from the coupler and place it over the end of the light guide (if it is not already installed on the light guide). Tighten with the setscrew. Place the light guide adapter into the Light Guide/Focusing Coupler and secure with the thumbscrew. The optimal position for the light guide in the Coupler, the position that transmits the most light, should be determined empirically.

For Filter Wheels internally mounted in the arc lamp: Insert one end of the Liquid Light Guide (or the end without a ferrule adapter) into the port next to the filter wheel. Secure with the set screw.

Insert the other end of the Liquid Light Guide (which may also have a ferrule adapter attached) into the Groony™ Light Guide adapter located in the back of the microscope. Make sure the Light Guide slides all the way into the Groony so that it rests just behind the cubes. Tighten the thumbscrew in the Groony to secure the Light Guide. If a LLG collimator or 3rd-party epi-fluorescence tube is provided, the LLG may be inserted into these parts without a ferrule adapter.

Connect the Sutter Controller to the Wheel using the DB15-DB15 cable in the Sutter box. When using the Sutter 10-2 Controller, connect this cable to the "Wheel A" port. Connect the Controller to the computer using the serial cable in the Sutter box. Use the color-coded COM port.

Connect all power cords to the surge suppresser (do not use the "Always On" receptacle if they are not needed). Please remember: DO NOT plug your Lamp into the surge suppresser -- use a separate wall receptacle.

BE CAREFUL NOT TO BEND THE LIQUID LIGHT GUIDE AT A SHARP ANGLE. Use wide turns only.

Step 7 -- Connecting the Camera

Unpack the Basler Camera and the Relay Lens. Remove the caps from the Camera and Relay Lens, and place them in a safe place.

Screw the relay lens into the camera using the threaded C-mount.

Remove the cover from the microscope phototube, and place it in a safe place. Insert the small end of the relay lens into the phototube and secure with the silver thumbscrew.

Use the USB-Micro B cable to connect the Camera to the Computer. The USB connection to the computer should be color-coded.

Step 8 -- Turning the System on

MAKE SURE THAT ALL COMPONENTS ARE TURNED OFF. MAKE SURE THAT THE XENON ILLUMINATOR IS PLUGGED DIRECTLY INTO THE WALL OR INTO A SEPARATE SURGE SUPPRESSER FROM THE OTHER COMPONENTS.

Turn on the Xenon Illuminator. Then turn on the surge suppressers for the other equipment.

Step 9 – Optimizing the light guide position

Using a fluorescence sample you can check to make sure that the light path in the microscope is optimized. First make sure that the light from the objective is approximately centered within the droplet containing the fluorescent indicator.

To check if the light path is optimized, go to **<Video Preview>** in the InCyt Im2™ program. Click on the **<Check Brightness>**. Increase the lamp intensity or exposure time until you see red (saturated) pixels on the screen. The first red pixels should appear in the center of the screen.

1. Move the objective up and down until the red area in the monitor is maximized. This ensures that the excitation light is focused within the droplet containing the fluorophore.

2. Loosen the set screw that secures the light guide adapter to the output side of the filter wheel. Slide the light guide in and out until the red area on the monitor is maximized. Lock the set screw.

Step 10 -- If you have any problems, do not hesitate to contact Intracellular Imaging or your distributor

III. IMPORTANT SAFEGUARDS

A. ELECTRICITY

- We recommend that you plug all system components, EXCEPT the light source, into the power strip. This will permit you to turn system components on and off with the power strip, rather than the individual component power switches, thus ensuring that everything is on or off together.
- Plug the light source into a separate power receptacle and turn it on and off with its own power switch only.
- **Turn on the light source FIRST, then turn on the other components. The light source is an arc lamp that generates 23,000 volts to fire. The RF interference from this spark could damage operating electronic equipment in the immediate vicinity. You may turn OFF components in any order.**

B. LIGHT SOURCE

- **Turn off any computers or electronic equipment in the immediate vicinity of the lamp before turning it on. RF interference during start-up could damage these devices if they are on.**
- **DO NOT look directly at the lamp**. The light produces high intensity visual and ultraviolet radiation that may cause burns to skin or eyes. UV goggles are provided for your protection. Use them anytime your eyes may be exposed to direct lamp light.
- DO NOT block air vents.
- DO NOT remove lamp housing or try to disassemble any part of the unit.
- Once you turn on the lamp, leave it on until it warms up completely (about 30 minutes) before turning it off. You may, however, use it immediately to view cells. If you turn the lamp off, DO NOT turn it back on until it has completely cooled -- we recommend at least 30 minutes. Turning on a warm lamp can result in poor performance and/or bulb damage.

C. SUTTER FILTER WHEEL/LIQUID LIGHT GUIDE

- Put on UV goggles before opening the filter wheel while the lamp is on.
- Be sure that all filters are fully screwed into the filter wheel before use.
- DO NOT expose the liquid light guide to direct light from the lamp. **Make sure there is always a filter or blank in all filter positions.**
- When not using the system or leaving the room for more than a few minutes, be sure to turn the filter wheel so that it is in a shuttered position or turn down the intensity of the lamp. This will prevent filter deterioration. While the filters in your system are designed to withstand high temperatures, continuous direct exposure to the 175-watt Xenon lamp will burn their coatings.

III. IMPORTANT SAFEGUARDS

D. COMPUTER WORKSTATION

- Close any other applications before starting the *InCyt Im2*™ or *InCyt Im1*™ software. These programs are very memory-intensive, and other open programs may impair performance.
- Be sure to save all images as soon as possible after completing an experiment. Images are written to a volatile portion of the hard drive (drive D:\) and **MUST** be saved to the D:\InCytIm2 directory (default when saving experiments) or another storage device before starting another experiment or turning the system off. Failure to do this may result in lost data.

E. GENERAL

- Experiments should be conducted in a moderately darkened room to prevent background light from affecting the quality of the images.
- Be sure to keep your system in a dust-free, temperature and humidity-controlled environment.
- Please read all accompanying component manuals for safeguards and cautions associated with each device.

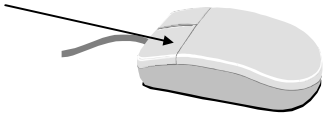
IV. KEY SYSTEM CONCEPTS AND PROCESSES

A. INTRODUCTION

The *InCyt Im2*™ program is easy to learn and use. The menu structure has been streamlined to contain only those capabilities and options that the user generally utilizes in the course of an experiment.

We have tried hard to make the user interface intuitive. When conducting an experiment, you move from left to right across the top menu bar. Wherever it is appropriate to do so, you are prompted to the next step by having those menu items that should not be selected yet "grayed out".

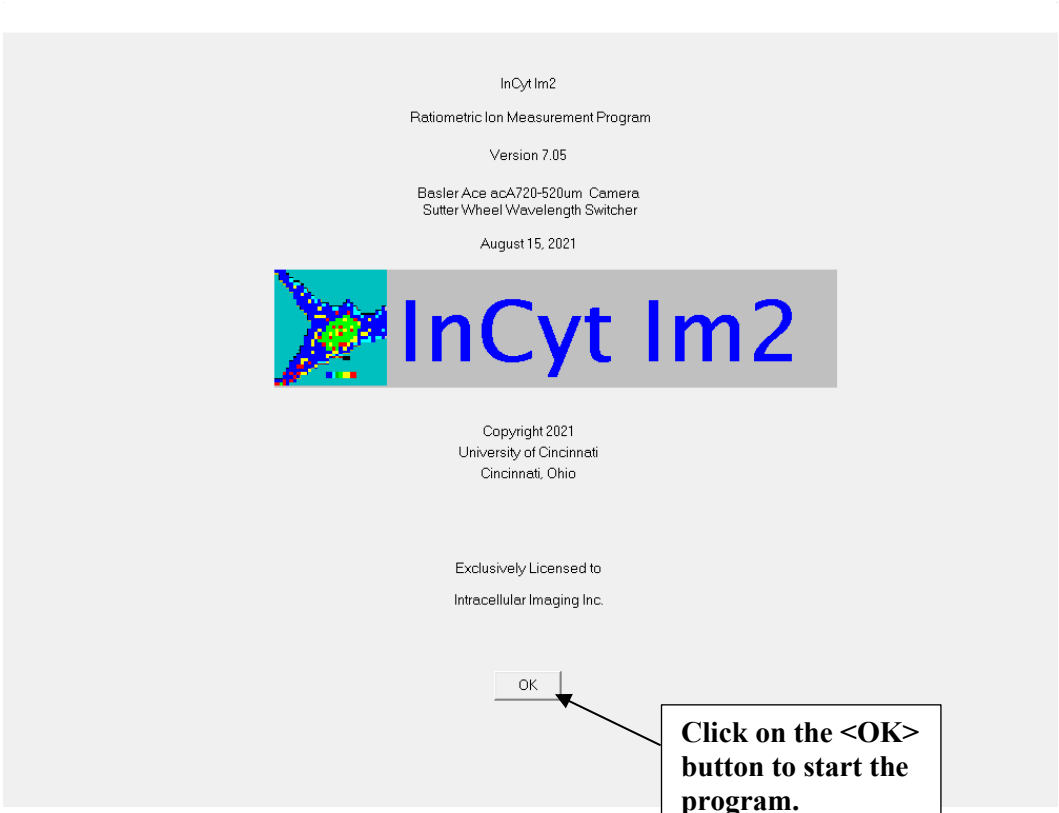
In the program description sections, brackets (< >) refer to a program menu item or button that can be accessed with a click of the LEFT mouse button.



Turning the System On

- Turn on the 175watt Xenon arc lamp FIRST.
- Then turn on the other system components.
- It is recommended that all components except the 175-watt Xenon arc lamp be connected to the same power strip so that all can be turned on with a single switch.

TO BEGIN THE PROGRAM, click on the *InCyt Im2*™ icon on your computer desktop. The following screen will appear:



IV. KEY SYSTEM CONCEPTS AND PROCESSES

B. MEMORY (RAM) IMPACT ON EXPERIMENTS

Your system has been delivered with at least 16 gigabytes of RAM (Random Access Memory). In setting up to do an experiment, the user has two choices of how to save images -- images can be saved either to RAM or directly to the hard disk. Saving to RAM allows the user to acquire data more quickly, but it places greater restrictions on the total number of images that can be gathered during the experiment. The system uses the Windows memory resources dynamically, so that image memory allocation is dependent on the amount of memory available at the time the program is executed. If the user chooses to save the images directly to disk, the user can capture up to 4,096 image pairs of any size. This may also be limited by the available disk space in the D:\ hard drive. When selecting to save to disk, the program takes a moment to allocate space on the D:\ drive to temporarily hold those images. This occurs at program start-up and after a change in the number of images to be saved in <Initial Settiings>.

C. CONTROLLING IMAGE BRIGHTNESS

Unlike other imaging systems, the *InCyt Im2*™ system gives you flexibility in controlling how bright your images are. This allows you to adjust for such factors as different cell types, dye-loading conditions, filter transmittance differences, changing bulb intensity, etc.

Lamp intensity and camera exposure time should be used together to achieve the optimal image. The lamp has an intensity knob on the front panel. The camera exposure time can be adjusted separately for each excitation wavelength in the **<Video Preview>** area of the program. The Basler ac720-520um camera used in the *InCyt Im2*™ system is an "integrating CCD" camera, meaning that it can integrate the exposure over the time set by the user. Exposure times can range from 1 millisecond/frame to 5 seconds/frame.

This camera cannot be damaged by overexposure, however, it is possible to saturate the camera with too much light. If the camera is saturated, it cannot discriminate changes in the fluorescent light intensity of the cells. You will want to make sure that your cells are bright enough for a good image, but not so bright that the camera is saturated.

D. CONVERTING FLUORESCENCE TO ION CONCENTRATIONS AND CREATING IMAGES.

The diagram on page 13 provides a graphical representation of this discussion. Dual-wavelength, or "ratio" experiments measure fluorescence intensity at two different wavelengths. These wavelengths are chosen based on how the dye absorbs and emits light. In calcium measurement studies, Fura-2 binds to free calcium ions (Ca^{2+}) in the cell. When Fura-2 molecules are NOT bound to Ca^{2+}, they absorb light maximally at a wavelength of 380nm and emit light at 510nm. When Fura-2 molecules are bound to Ca^{2+}, they absorb light maximally at 340nm and emit light at 510nm. The 510nm emissions are captured by the camera as a black & white image. By measuring the ratio of the two emission intensities for excitation at 340nm and 380nm, Ca^{2+} concentration can be calculated in any given area.

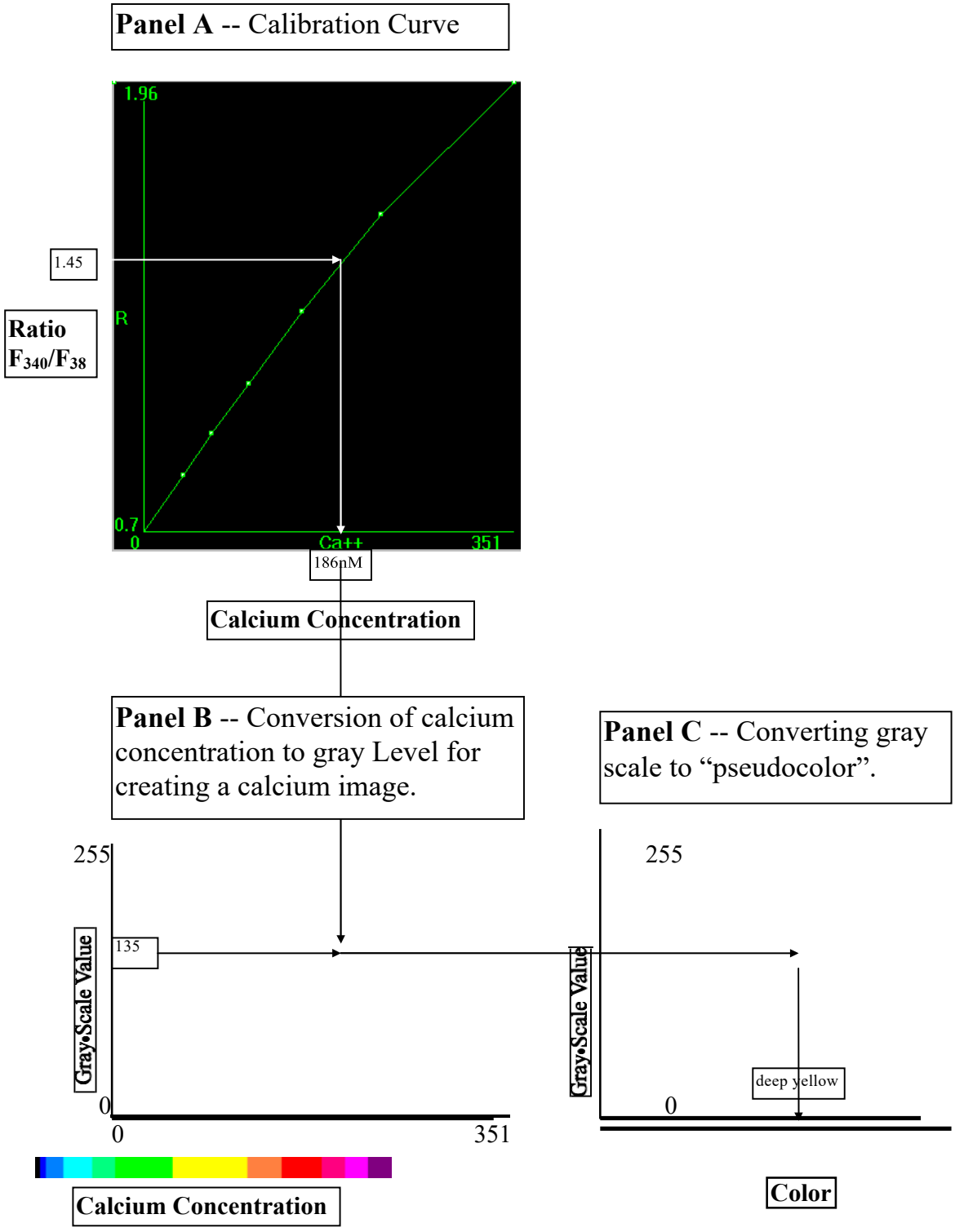
IV. KEY SYSTEM CONCEPTS AND PROCESSES

The first step (Panel A on the next page) is to "teach" the system what ratio of 340nm-to-380nm emission corresponds to each calcium level. This is often done with calcium standard solutions, which are used to create a graph of fluorescence ratio (F_{340}/F_{380}) as a function of Ca^{2+} concentration. This graph is then used to convert fluorescence ratios in an experiment to calcium concentrations.

A "ratioed" image is then developed by assigning each Ca^{2+} level one of 256 gray-scale values (Panel B). The lower calcium concentrations are given darker values, and the higher calcium levels are given lighter values. Because it is easier for most users to distinguish colors rather than gray-scale values, the user can assign colors to different gray scale values (Panel C -- for more information about assigning colors, see **<Psuedocolor>** under **<Utilities>** in Section V). For example, blues and greens can be assigned to darker gray-scale values and oranges and reds can be assigned to lighter gray-scale values.

IV. KEY SYSTEM CONCEPTS AND PROCESSES

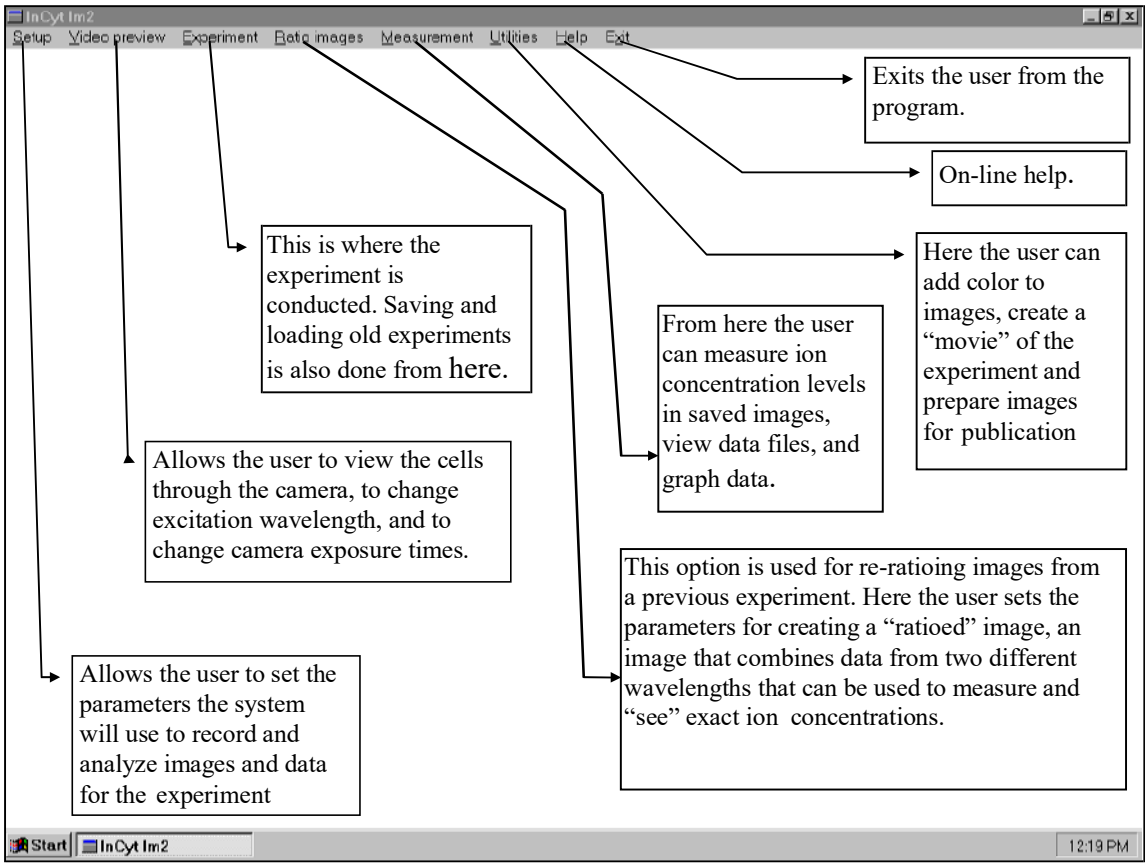
D. CONVERTING FLUORESCENCE TO ION CONCENTRATIONS AND CREATING IMAGES (continued)



V. *InCyt Im2*™ PROGRAM: Main Menu

A. MAIN MENU

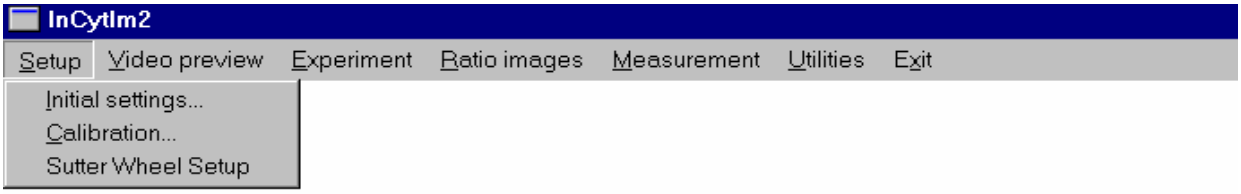
The user moves left to right through these menu items to run an experiment.



For simplicity reasons, the following discussion of the program will assume that you are running calcium studies using the dye Fura-2 and will refer to measuring "ion concentrations". The *InCyt Im2*™ program is extremely versatile and can be used to measure a range of other ions, as well as other fluorescent indicators. If you have questions about how to use the system for your research, please call us.

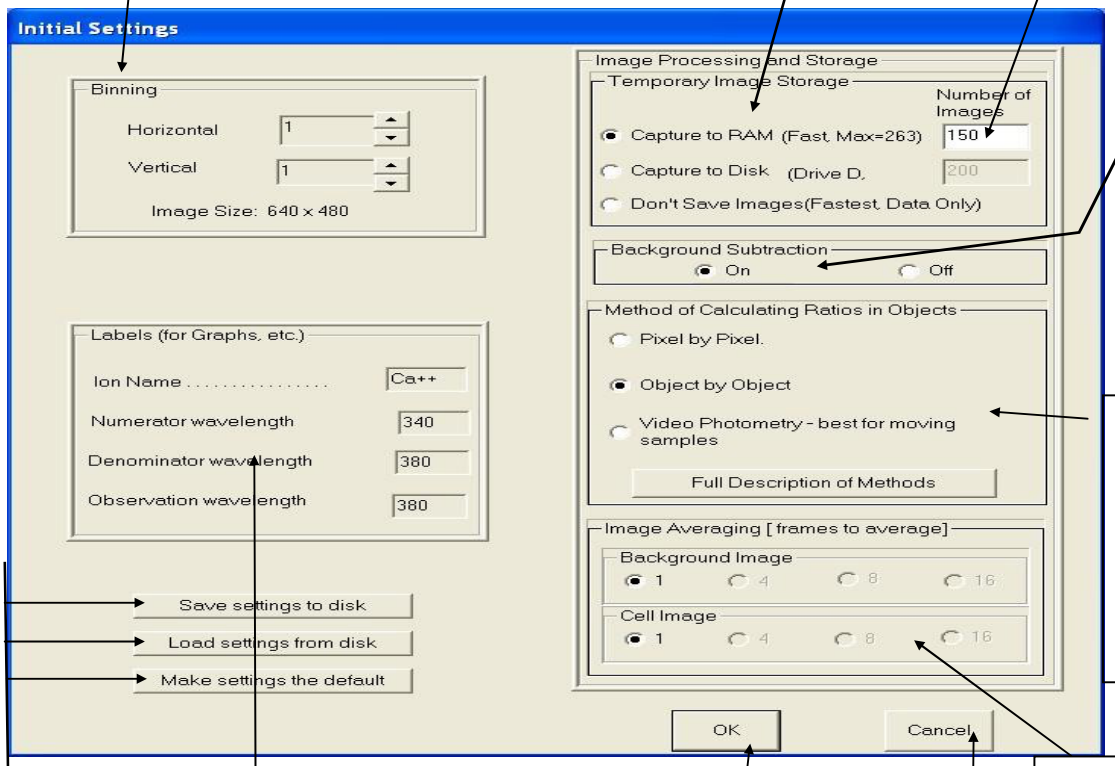
V. *InCyt Im2*™ PROGRAM: Setup > Initial Settings

B. SETUP



1. Initial Settings

The **<Initial Settings>** dialog box is used to select the size and number of video frames that will be captured during an experiment. It is also used for selecting the method for calculating fluorescence ratios (pixel-by-pixel or object-by-object) and for selecting whether image averaging will be implemented.



Click on the radio buttons to set the number of pixels to be binned. As binning increases, the size of the image decreases and the maximum number of images saved to RAM (indicated by the next box to the right) will increase. The brightness of the image also increases.

Choose how you want to save images, to RAM or directly to disk. Then type the number of image pairs you want to collect in the box.

Choose whether or not to subtract background light from the cell images.

<Object by Object> is recommended unless you will need to image average or your samples are moving.

Creates and recalls settings chosen on this screen.

These labels are carried to other program screens, data files, and printouts, but have no other function. They are selected in <**Sutter Wheel Setup**>.

Approve settings and exit.

Exit without changing original settings.

Choose how many images to average to produce a single display image. Image averaging will slow data collection, but reduces image noise.

V. *InCyt Im2*™ PROGRAM: Setup > Initial Settings

1. Initial Settings

1.a. Frame Size

Binning works by combining neighboring pixels. It therefore affects the size of the image and the resolution of the image, but not the field covered by the image. Thus, when you increase the binning, you will see the same number of cells and they will be brighter, but there will be less detail. Because less binning takes more memory, the binning selection will determine how many image pairs you can acquire in RAM if you are saving images for later analysis. A full size image has somewhat more than 600,000 bytes of data, or 600KB, so that the amount of RAM required to save all three images at a single time point (340nm, 380nm, and the ratioed image) is nearly 2MB.

Choose the frame size that best allows you to both capture the number of cells you want to see and allows you to capture the number of images you need.

1.b. Number of Images

If you choose to **<Save Images to Disk>**, you can acquire image pairs for almost as many time points as you want for any size image. The images will be temporarily saved to the D:\ drive while the experiment is running. Type in the number of images you think you will need for your experiment in the entry box. You may want to type in a number that is a little larger than the number you expect you will need, because the experiment may run longer than you anticipated. You can always stop the experiment if it is completed before all images are taken. Once the experiment is completed, you will be given a choice of whether to change the images from their temporary storage to permanent storage.

NOTE: It takes the system a moment to allocate file space for the images on the D:\ drive. The more you select, the longer it takes. The system will return to the **<Main Menu>** when this task has been completed.

The fastest way of acquiring images is to **<Save Images to RAM>**. However, the number of images you can collect is limited by the amount of unoccupied RAM in your workstation.

The maximum number of image pairs the system will allow you to collect is displayed below the entry box. Type in the number that you wish to acquire during the experiment up to the maximum number displayed. Once again, you may wish to type in a number somewhat larger than what you expect you will need.

If your experiment runs longer than the number of images you have selected, the experiment will continue to collect data, but will stop collecting images beyond the number you specify in <**Initial Settings**>.

PLEASE NOTE: If you load an experiment into the **<Measurement>** section of the program, it will automatically reset your selected number in **<Initial Settings>** of images to the number in that experiment. If you wish to run a new experiment before exiting the program, please return to **<Initial Settings>** and reset the desired number of images.

V. *InCyt Im2*™ PROGRAM: Setup > Initial Settings

1.c. Labels

These fields do not have a direct functional effect on the experiment. However, it is important to properly label the experiment. These labels serve as a record of the wavelengths and ion studied; they will be used to mark data files, graphs, and other system outputs. These label settings are established under **<Sutter Wheel Setup>** and carried over t this screen.

1) **<Ion Name>**: enter the name of the ion or other cell indicator that is the experimental focus.
2) **<Numerator Wavelength is>**: This wavelength should be the one that shows an increase in emission intensity as the concentration of the ion increases (e.g., 340nm for Fura-2). Enter the wavelength in nanometers.
3) **<Denominator Wavelength is>**: This wavelength should be the one that shows a decrease in emission intensity as the concentration of the ion increases (e.g., 380nm for Fura-2). Enter the wavelength in nanometers.

1.d. Background Subtraction

There is generally a certain amount of background noise associated with a measurement. The source may be the result of thermal noise in the camera, ambient light in the room, auto fluorescence in the cell medium, or trace amounts of dye not fully flushed after loading. If you choose to subtract the background, you will be given the opportunity to determine the background level just before starting the experiment.

The background light level is subtracted from the image light level on a pixel-by-pixel basis. Therefore, if there is more background light in one area of the field of view, more light will be subtracted from the image in that area. What will remain in the image is only that fluorescence that is higher than the background level. Therefore, the signal-to-noise ratio of the image is improved.

In general, Intracellular Imaging recommends subtracting background. Choose **<No>** here only if you are certain that your background levels light are extremely low or you have some other compelling reason.

V. *InCyt Im2*™ PROGRAM: Setup > Initial Settings

1.e. Method of Calculating Ratio

1) **<Pixel by Pixel**>: In this method, background at each wavelength is subtracted on a pixel by pixel basis, and a fluorescence ratio is then calculated for each pixel. Finally, the average ion concentration within an object is calculated from the average of all the pixel ratios. This method gives equal weight to pixels from thick (brighter) regions of the cell and thin (fainter) regions because the ratio is calculated for each pixel and the calcium is then calculated from each of those ratios.

2) **<Object by Object>**: In this method, background at each wavelength is measured on a pixel by pixel basis. The average fluorescence of all the pixels within an object is then calculated for each of the two wavelengths. The ion concentration within an object is then calculated from the ratio of these two average fluorescences. This method gives more weight to thicker (brighter) regions of the cell than to thinner (fainter) regions because these regions contribute more to the total fluorescence and the ratio is calculated from the total fluorescences at 340 and 380, respectively. This method may also produce more accurate results when the fluorescence signal is very weak.

3) **<Video Photometry>**: In this method, each object is treated like a single, large pixel. The total fluorescence from all the pixels in the object are added together at each wavelength. Total background levels from each object are then subtracted from these total fluorescence levels. The average ion concentration in the object is calculated from the ratio of these two total fluorescence numbers. This method is best for moving objects, such as blood vessels. Thresholding is not allowed with this method.

1.f. Image Averaging

If you choose the **<Pixel by Pixel>** method of calculating ratios, you have the option of taking multiple images to create an 'averaged' image for analysis. This helps to further reduce noise in the image, providing a cleaner image and a smoother background. You can use multiple images to create both an average background and average experimental images. You have the option of averaging 4, 8, or 16 images for each saved image. If you choose the option **<1>** here, image averaging is effectively turned off. (Image averaging is not needed (or allowed) with object by object measurements because the noise is effectively averaged over each object.)

The tradeoff with image averaging is that in order to reduce noise, it takes longer to generate each image. For example, if your exposure times are 200 milliseconds (0.2 second) at the 340nm wavelength and 100 milliseconds at 380nm, without image averaging the system will be able to acquire about 68 images per minute. If you choose to average across 16 images, the system will be able to acquire only about 7 saved images per minute, or one every 8.6 seconds. For some experiments, this may be too slow. As a general rule, if you are image averaging, you should average 16 background images, since the time it takes for background capture is not usually an inconvenience. You may then wish to average only 4 or 8 images during the experiment.

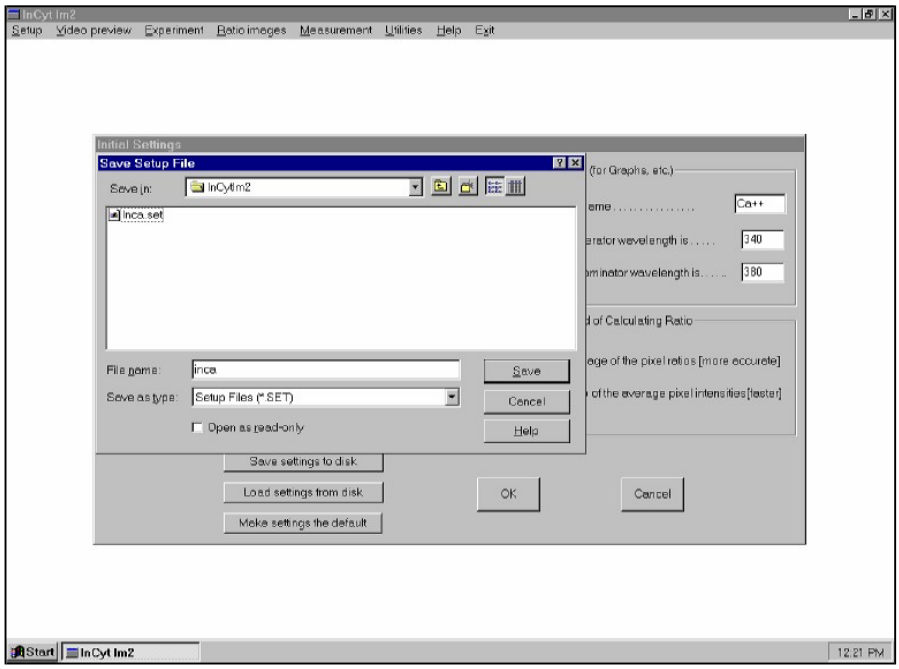
Choose the level of image averaging based on the acceptable noise level and the response time of your cells.

V. *InCyt Im2*™ PROGRAM: Setup > Initial Settings

1.g. <Save Settings To Disk> <Load Settings From Disk> <Make Settings The Default>

These options will save the Initial Settings in a tab-delimited ASCII file with a "**.set**" extension. Multiple users can save and reload their Initial Settings preferences without having to reselect them for every experiment. When you select **<Save Settings To Disk>**, a standard Windows file dialog box will prompt you to name the settings file as shown below. If you want any selection of settings to come up every time you start the program, hit **<Make Settings The Default>.** Default settings are saved in the file **"incytim2.set"**.

PLEASE NOTE: If the program begins to behave erratically, it is possible that the setup file (a text file) has been corrupted or altered in a way that does not allow the program to set up properly. If this happens, you may need to restore the original **incytim2.set** file from your program backup. This file is placed in the C:\InCytIm2 directory.



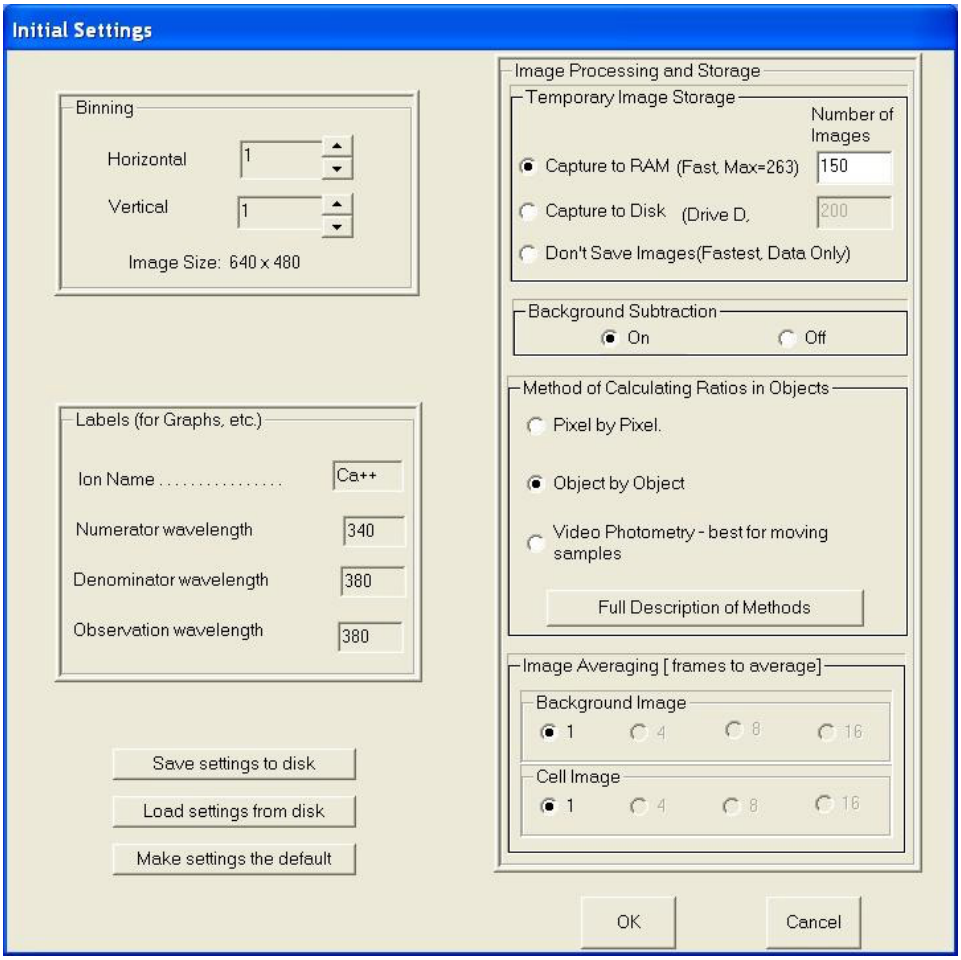
1.h. <OK> and <Cancel>

You must click on one of these buttons to exit the Initial Settings Screen. If you approve of the settings you have chosen, then click on **<OK>** and the settings will be held in memory for the experiment. **<OK>** does NOT save the settings to a file. If you exit the program, the settings will revert to the "Default" settings file when you reopen the program.

<Cancel> ignores any changes you have made to the settings screen and brings you back to the Main Menu.

V. *InCyt Im2*™ PROGRAM: Setup > Initial Settings

The examples we will use for most of the discussion in this manual are from an experiment titled "**eg1216#2**". The following are the Initial Settings from this experiment.



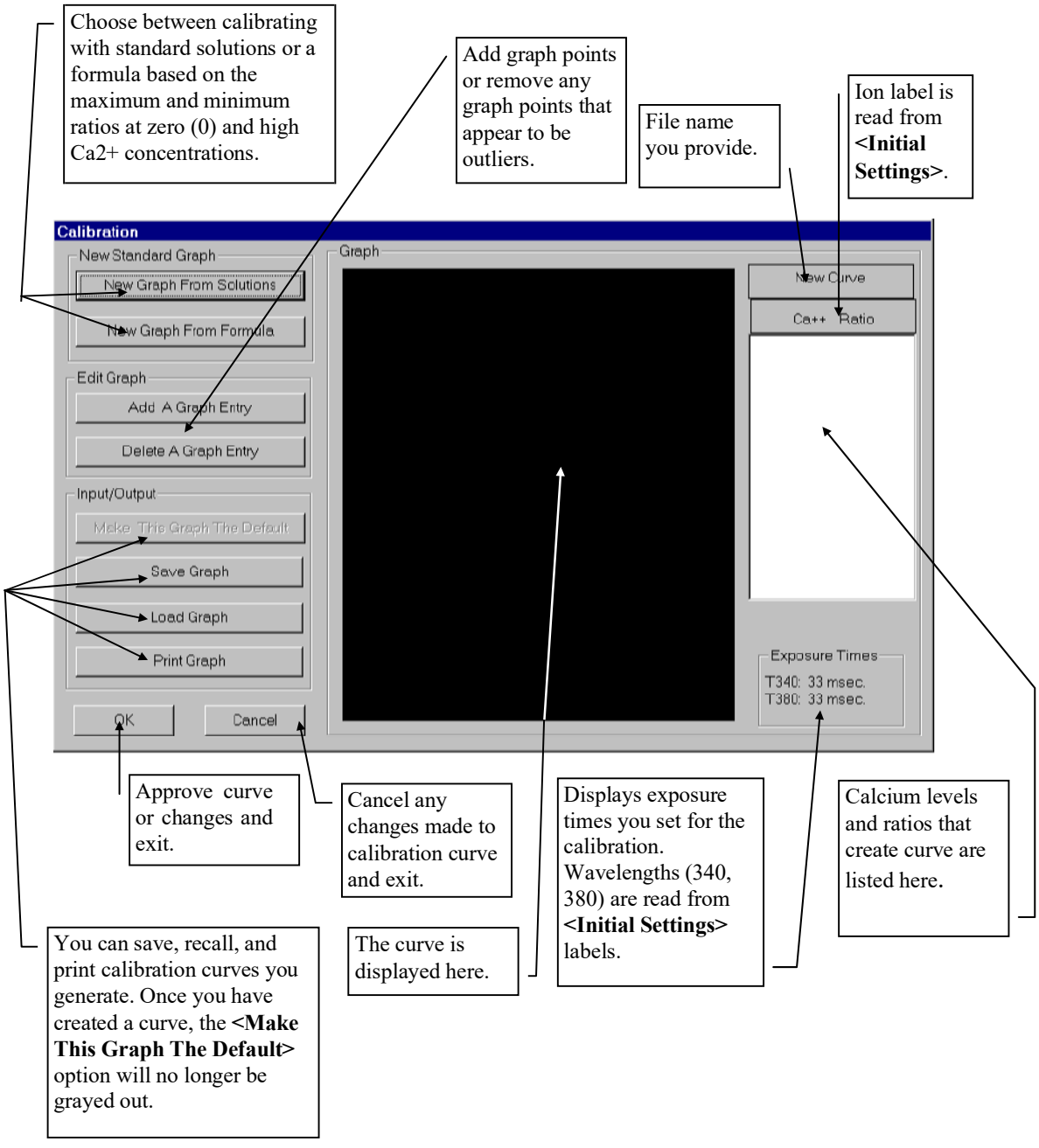
These settings indicate:

1) Full-size images (480 x 640 pixels)
2) Images will be saved to RAM. Although the system could save up to 263 image pairs, only 150 will be collected in this experiment.
3) Ca^{2+} is being measured using a dye that fluoresces at 340nm and 380nm. The signal at 340nm increases as Ca^{2+} levels increase.
4) Background noise will be subtracted.
5) In calculating 340nm/380nm ratios (and therefore Ca^{2+} levels) for an object, the total fluorescence at 340 and the total fluorescence at 380 will be calculated and then a single ration will be calculated and converted to calcium. (This option does not allow the user to image average, as indicated by the graying out of the Image Averaging options.)

V. *InCyt Im2*™ PROGRAM: Setup > Calibration

2. Calibration

After you complete the Initial Settings, move to **<Calibration>** within the **<Setup>** menu. The calibration curve you develop here establishes the relationship between the ratio of fluorescence intensities at the two wavelengths and the Ca^{2+} concentration. In developing the calibration curve, you will set your lamp intensity and the camera exposure/integration time at each wavelength independently.



V. *InCyt Im2*™ PROGRAM: Setup > Calibration

There are two ways of creating the calibration curve:

1. From a set of solutions that have known Ca^{2+} concentrations. These solutions are available commercially. It is assumed that the dye has the same fluorescence properties inside the cells as it does in solution. While the dye does behave somewhat differently inside the cell than out, the differences (at least for Fura-2 and Ca^{2+}) are small enough to ignore in most experimental situations. This method can be used before the experiment begins.

2. From the actual minimum and maximum possible Ca^{2+} levels inside the cells. Some experimental protocols require that the calibration reflect the behavior of the dye within the particular cell line. This method generates the end-points of the graph -- a standard formula then calculates the shape of the curve (see Grynkiewicz G., M. Ponce, and R.Y. Tsien, "A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, Journal of Biological Chemistry, 260 3440 (1985)). If you need to generate this graph with the exact same cells you run the experiment with, you must generate the curve after the experiment is completed. Alternatively, you could generate your calibration with a separate set of cells before the experiment.

a. Creating a <New Graph From Solutions>, or "Standard Curve"

Even if you decide to create a graph from a formula based on the dye's behavior in the cells themselves, we recommend that you periodically generate a calibration curve from solutions. Solutions allow you to become familiar with what sort of ratios you should expect at a number of different Ca^{2+} levels. They also give you a consistent barometer with which to test the performance of your system. Lamps and filters can degrade over time, and this can be identified by tracking how the system measures standard solutions. Finally, standard solutions allow you to quickly begin experiments on cell lines that are unfamiliar.

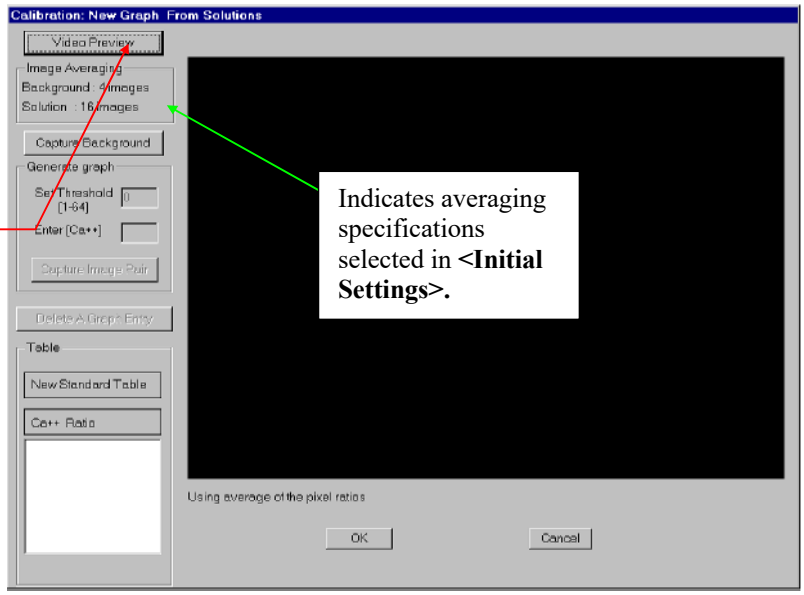
You can use the standards dish supplied with your system to hold up to 6 different standard solutions in a single dish. Calcium standards can be obtained as a kit from Molecular Probes containing the following concentrations: 0 (no Ca^{2+}), 38nM, 65nM, 100nM, 150nM, 225nM, 351nM, and 602nMm.

NOTE:	The Appendix also contains a complete discussion of generating a Standard Curve. It is recommended that when generating a Standard Curve, you temporarily set the **<Frame Size>** to quarter-size (240x320). This will collect data from the center of the field, which has the most even illumination.

Click on the **<New Graph from Solutions>** button.

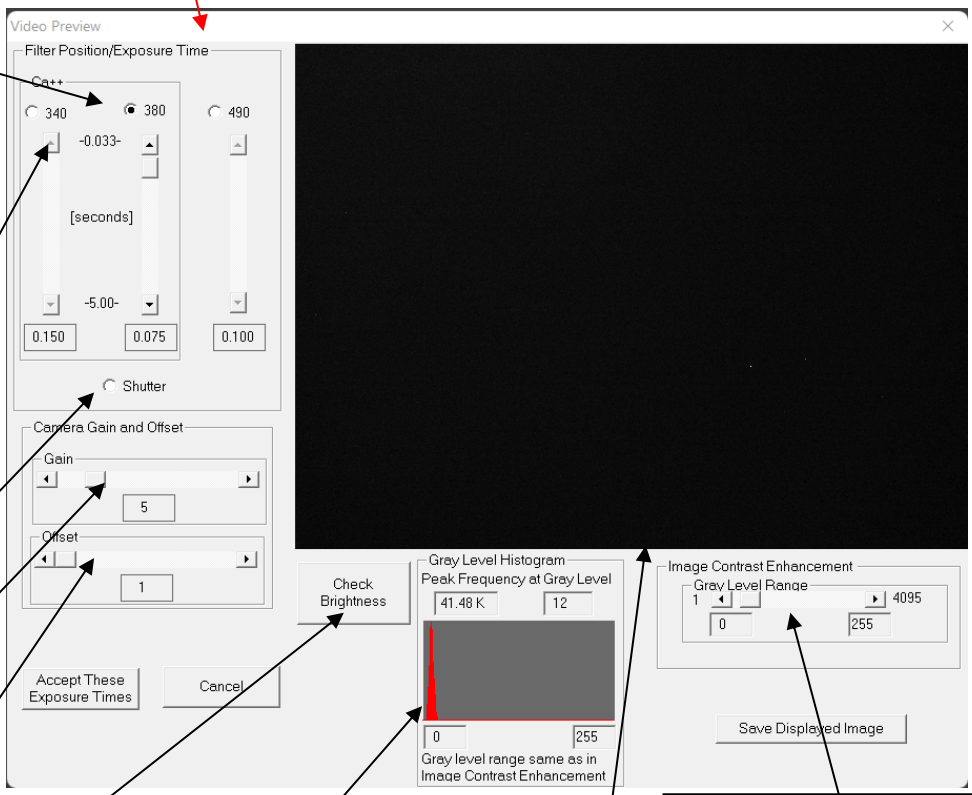
The graph-generation screen (shown next page). With this screen and those for other multi-step processes, the program prompts the user through the steps by highlighting only those steps that are available to the user at any given time. Move from the top to the bottom through the screen.

V. *InCyt Im2*™ PROGRAM: Setup > Calibration



STEP 1: Go to **<Video Preview>** to set lamp intensity and exposure times.

Indicates averaging specifications selected in **<Initial Settings>.**



Radio buttons switch filter wheel among filters.

STEP 2: Set exposure times at 340nm and 380nm excitation using up and down arrows. (see below)

Engage shutter if the system is to be left for more than a few minutes to protect filters.

Increasing camera Gain will make the camera more sensitive and the image brighter, but it may increase noise in the image. Offset is rarely used. It will increase the ability to measure the faintest signals.

This allows you to check if any part of the image is too bright (saturated) or too dark. A saturated pixel is RED; a black pixel is GREEN. When **<Check Brightness>** is on, this button will read **<Reset to Gray>**.

The histogram shows the range of pixel intensity. For standard solutions, the histogram is usually a narrow bell-shaped curve.

Video image is presented here. When viewing standard solutions, this will be a solid field between gray and white.

Slider is used to adjust dynamic range of the display. Lower values will cause image to appear brighter. This feature only affects the image on the monitor, not the data being collected. The upper limit is typically set to well below 4095 (indicating which of the camara's 12 bits of dynamic range will display as saturated.

V. *InCyt Im2*™ PROGRAM: Setup > Calibration

Step 2 -- Setting Video Exposure/Integration Times and Lamp Intensity.

DARKEN THE ROOM. Place the 100nM standard solution (containing Fura Acid -- see Appendix) on the stage and adjust the objective until the spot is as sharp as possible. (You may wish to use the "Check Brightness" option to help you do this by getting as many red pixels as possible.) Set the filter to the 380 position. Turn the lamp intensity dial up until you can see a green fluorescent spot in the 100nM droplet. This spot should be centered so it does not hit the edges of the droplet, causing large amounts of refraction. Adjust the exposure times for the 340nm and 380nm image so that to your eye the image on the screen appears to be roughly about the same brightness at both wavelengths. Use the radio buttons at the top of each bar to switch between wavelengths.

The exposure times can vary between 0.001 seconds (1 millisecond) and 5 seconds. Start off with 380nm at a relatively short exposure time (e.g., 0.100 seconds). The image on the screen should be a medium to light gray. If the image is white, either drop the exposure time or turn down the lamp. If it is very dark, increase the exposure time or turn up the lamp. (Remember to switch the microscope to "Photo", so that the camera can see the image. The exposure times you set will affect how quickly you can acquire images during the experiment.)

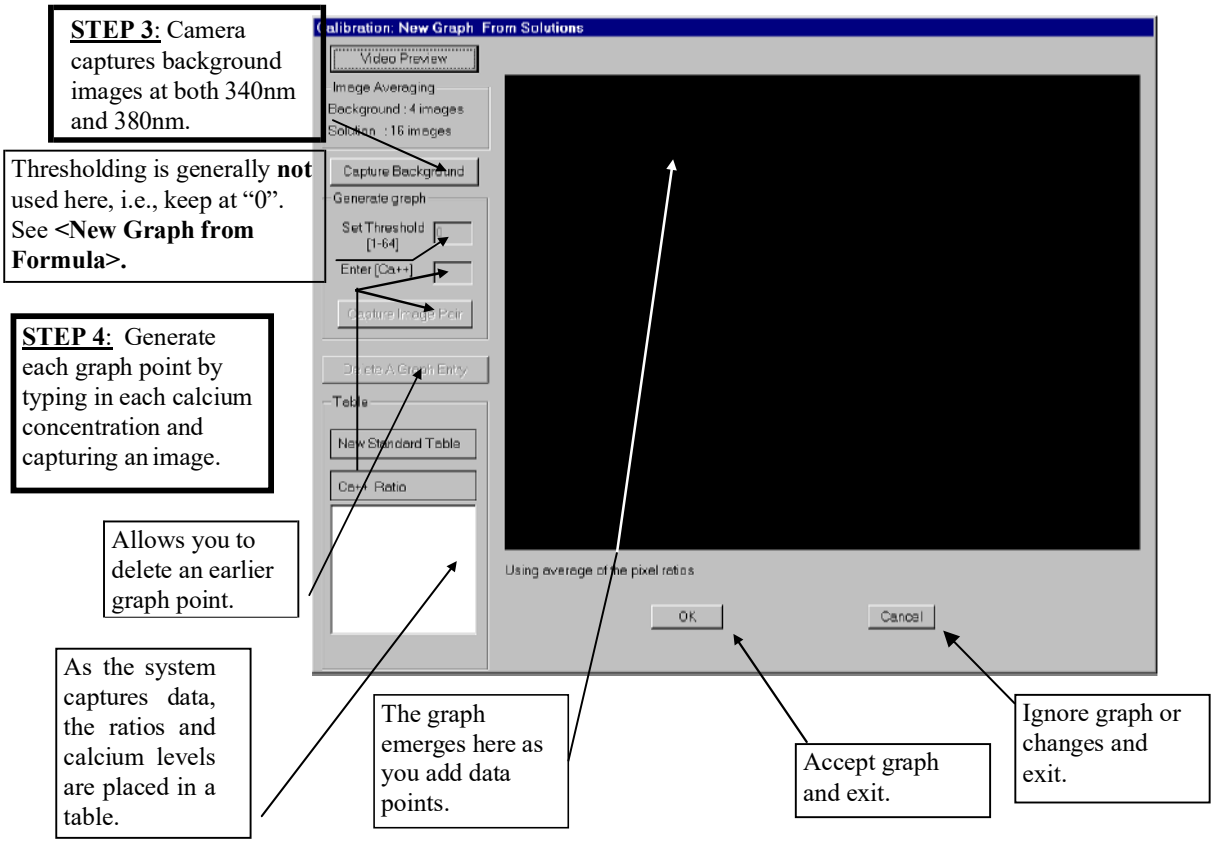
The system will often produce less light intensity at 340nm than at 380nm. Therefore, you may need to lengthen the exposure time at 340nm relative to 380nm to get equal brightness between the two wavelengths with the 100nM solution. The ratio of the 340nm to 380nm exposure time is called the "**integration ratio**". To get equal intensities from a 100nM Ca^{2+} solution, you should require somewhere between a 1:1 to a 5:1 ratio. Over time, this ratio will start to creep up, because as the lamp degrades, it loses 340nm intensity first. For our example above, the integration times were set at 0.150 seconds for 340nm and 0.075 seconds for 380nm, for an integration ratio of 2:1.

Once the integration times are set so that for 100nM Ca^{2+} the light intensity on the monitor does not vary a great deal as you switch between wavelengths, place the 0nM calcium solution over the objective. Switch the wavelength to 380nm. The 380nm image for the standard curve will never get brighter than it is right now, nor will the 340 signal get fainter. Make sure that the intensity at 340 is noticeably greater than the background intensity, otherwise the signal to noise ratio will be too low to give a good standard curve. If it is too low, increase the lamp brightness but do not change the exposure times.

As a final check on lamp intensity, place the solution with the highest calcium level (e.g., 602nM) over the objective. Switch the wavelength to 340nm and **<Check Brightness>** for saturated pixels again. The 340nm intensity is brightest at these high calcium levels while the 380 image is faint. If the 380 image is not appreciably above background, increase the lamp intensity.

To accept these exposure times, click on **<OK>** to exit **<Video Preview>** and return to **<New Graph From Solutions>**.

V. *InCyt Im2*™ PROGRAM: Setup > Calibration



Step 3 -- Capturing a Background

Before clicking this button, defocus the microscope or remove the standards dish from the scope so that you are capturing a non-fluorescent field. Both a 340nm and 380nm background are captured, since the exposure times for the two wavelengths may be different.

Step 4 -- Generating the Graph

Refocus the microscope on the 0nM solution. Type "0" in the **<Enter [Ca++]>** box **(NOTE: Always start with the lowest ion value first…otherwise the program may shut down prematurely.)** and hit the **<Capture Image Pair>** button. The system takes both images, averages images, and calculates the ratio. The calcium concentration and calculated ratio are written in the box at the lower-left hand corner of the screen. The ratio presented is equal to:

(340nm fluorescence intensity - 340 background)/
(380nm fluorescence intensity - 380 background)

The 340nm intensity is in the numerator of this equation -- this is what you indicated when you entered "340" in the **<Initial Settings> <Labels>** field **<Numerator Wavelength is>**. Because you set the exposure times to yield equal light intensity between the two wavelengths at 100nM, you should get a ratio of somewhere around 1:1 for the 100 nM solution. In that case, the ratio will be below 1.00 for calcium values below 100nM and above 1.00 for calcium above 100nM.

V. *InCyt Im2*™ PROGRAM: Setup > Calibration

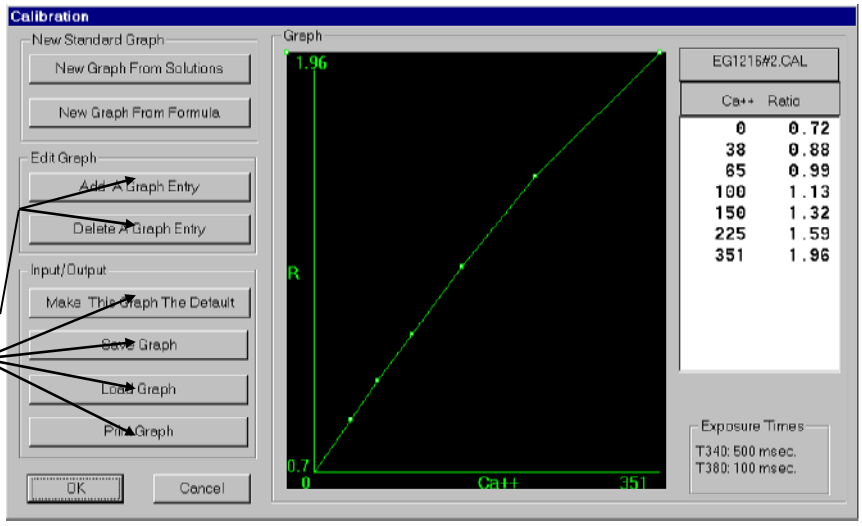
Step 4 -- Generating the Graph (continued)

Next place the 38nM solution (or next highest concentration available) on the microscope. Type "38" in the **<Enter [Ca++]>** box and hit the button again. Now a two-point graph will appear in the large box in the center. Continue with all the solutions. The system expects a higher ratio as the calcium concentration of the solution increases. If it does not see this, the system will give an error message stating that the Ca2+ concentration and ratio should increase monotonically.

Hit **<OK>** when finished with all of the solutions and you are satisfied with the graph. Your graph should look something like the picture below.

Add or delete graph points. To delete, highlight the problem point on the chart to the right then click on **<Delete a Graph Entry>**. To add a new value, the system will provide a dialog box for the new point after you click on **<Add a Graph Entry>**.

Manage **".cal"** files created to hold calibration information



The following are things you should look for in your calibration curve:

1) The ratio at 100nM calcium (or whatever concentration level of another ion you used to set exposure times to generate equal light intensity) should be approximately 1.00.

2) While your curve does not need to be perfectly smooth, any clear discontinuities are probably an indication that something is wrong with the solution. Try to take another measurement of the questionable solution. On the previous screen, you can do this by highlighting the problem point in the table and hitting the **<Delete a Graph Entry>** button. Then reenter the calcium concentration level into the **<Enter [Ca++]>** box and hit the **<Capture Image Pair>** button again. If a large discontinuity still exists at this point, you can just delete the graph entry in either of the two calibration screens.

V. *InCyt Im2*™ PROGRAM: Setup > Calibration

Step 4 -- Generating the Graph (continued)

3) Check the "dynamic range" of the ratio values. A wide range means the system will be able to more accurately distinguish Ca^{2+} changes. The above range of 2.72 (1.96/0.72) is acceptable, but look for a dynamic range of around 3.00 from 0nM to 351nM standards, and a greater range if using a 602nM solution.

> **NOTE**: The program interpolates between points used to develop the graph in **<Calibration>**. If the system registers a ratio higher than that used in the calibration, the last two known points of the graph are used to extrapolate the graph. Areas with ratios below that registered for 0nM calcium in the calibration are assigned a value of 0nM.

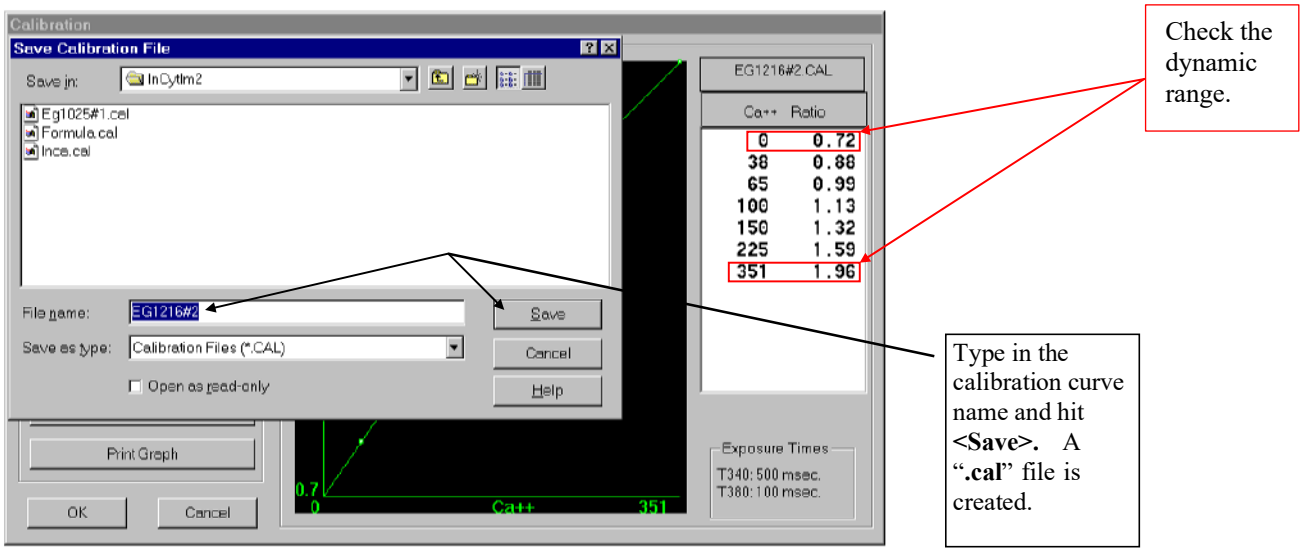
Step 5 -- Print and/or Save the Graph

If you are satisfied with your graph, please save your work.

<OK> will keep the graph in memory, but not save it. The graph will be lost when you turn off the machine or **<Load Graph>.**

<Make This Graph the Default> saves the graph name in the file **"incytim2.set"**. The default graph automatically loads when the program starts.

<Save Graph> gives you the opportunity to save the graph data for future experiments under a name you choose. The following dialog box appears. **NOTE: Do not put any spaces in the calibration file name.**



<Load Graph> allows you to recall a graph saved earlier.
<Print Graph> prints the graph, data table, filename, and exposure times.
<OK> saves the graph in memory and brings you back to the **<Main Menu>**.
<Cancel> exits the Calibration screen without saving any of your work. Be careful.

V. *InCyt Im2*™ PROGRAM: Setup > Calibration

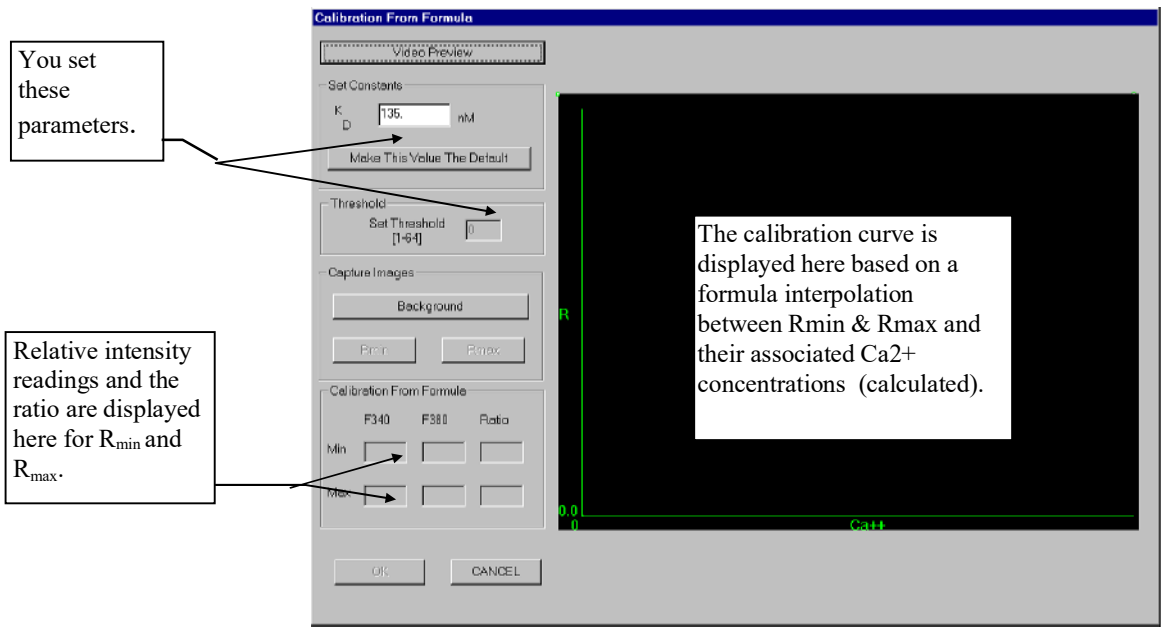
b. Creating a <New Graph From Formula>

Another way to generate a calibration curve is by a formula of three terms: R_{min} (the ratio at minimum Ca^{2+} levels), R_{max} (the ratio at maximum, or saturated Ca^{2+} levels), and the dissociation constant K_D. This formula is explained in more detail by Grynkiewicz G., M. Ponce, and R.Y. Tsien, "A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, Journal of Biological Chemistry, 260 3440 (1985). R_{min} and R_{max} can be measured either from two standard solutions (e.g., 0nM & 1mM, or higher) or from minimum and maximum calcium levels in the cells themselves. R_{min} and R_{max} form the two the two endpoints of the curve and the system interpolates the remaining curve from the formula.

If R_{min} and R_{max} are to be measured within the cells, it is necessary to permeabalize the cells to calcium in order to obtain the required values. The advantage of this method is that it is sensitive to the chemical state of the intracellular Fura-2. For example, incompletely hydrolyzed Fura-2 or photodamaged Fura-2 will affect the ratio observed at any given Ca^{2+} level, and therefore affect the shape of the true calibration curve. In these situations, the curve developed with calcium standard solutions could give less accurate Ca^{2+} readings in the cell.

Before you start the experiment, you need to provide the system a calibration curve. A formula-based curve can be generated from low and high calcium standard solutions or by reserving one plate of cells to generate the curve. If you wish to measure R_{min} and R_{max} in the experimental sample, these calculations must be done after the experiment is completed. Therefore, you must use a preliminary calibration curve, save the images from the experiment then generate a new curve from the experimental sample.

Hit the button **<New Graph From Formula>** in the first **<Calibration>** screen.



V. *InCyt Im2*™ PROGRAM: Setup > Calibration

<Video Preview> Use to position the field of view to image selected cell areas. To help you limit the field of view, you can go back to initial settings and reduce the frame size.

<Set Constants> Input the K_D constant in the dialog box. Typically this value is approximately 225nM for Fura-2 cytoplasmic ion conditions. But refer to the Molecular Probes catalog for more details.

<Set Threshold> Set this threshold to the same level you used or will be using during the experiment (see **<Experiment>** for a complete discussion of thresholding).

<Capture Background> Employs the same method as described in **<New Graph from Solutions>**, described in the previous section. Remember to defocus the microscope before taking the background image.

<R_{min}> If building the formula with standard solutions, focus on a 0nM calcium solution. If conducting an *in situ* measurement, add calcium-free extracellular buffer containing a calcium ionophore (e.g., ionomycine or BrA23187) plus sufficient EGTA to bring the [Ca^{2+}] to zero. Now click on **<R_{min}>**.

<R_{max}> If building the formula with standard solutions, focus on a calcium solution of 100 µM or greater. If conducting an *in situ* measurement, add sufficient Ca^{2+} back to the extracellular buffer containing the ionophore to overwhelm the EGTA and to give a final concentration of free Ca^{2+} in the cells of greater than 100 µM.

NOTE: **<R_{min}>** and **<R_{max}>** readings can be taken in any order.

<OK> Saves the graph in memory and brings you back to the first **<Calibration>** screen.

<Cancel> Exits this screen without saving any of your work. Be careful.

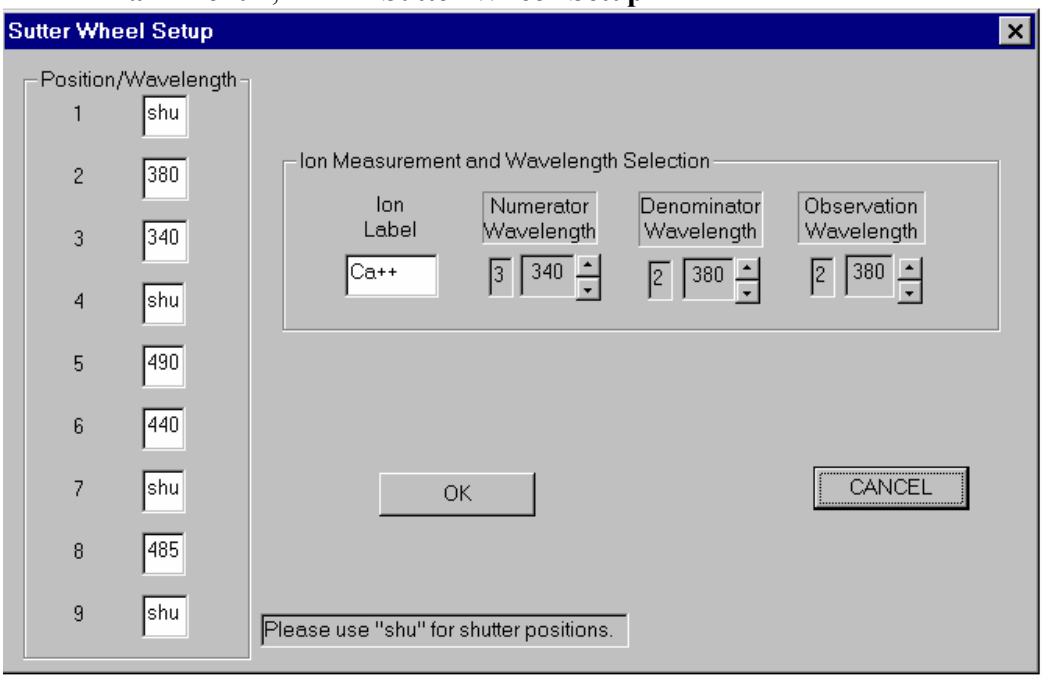
PLEASE NOTE: Intracellular Imaging has found that the relative strength of the 340nm and 380nm signal can vary based on the light intensity. Therefore, a Calibration Curve may be accurate for one lamp intensity setting and not for another. In general we recommend that you keep the lamp intensity the same as much as possible. If you find that you need to change the lamp intensity significantly for a new set of experiments, we recommend that you run another Calibration Curve to check that the relative 340nm and 380nm signals have not changed.

V. *InCyt Im2*™ PROGRAM: Setup > Sutter Wheel Setup

3. Sutter Wheel Setup

a. Program notes

Your Sutter Wheel has been loaded with the filters you ordered. Please refer to the Sutter user's manual if you need additional filters with the wheel. Under the <**Setup>** section of the **<Main Menu>**, select **<Sutter Wheel Setup>**.



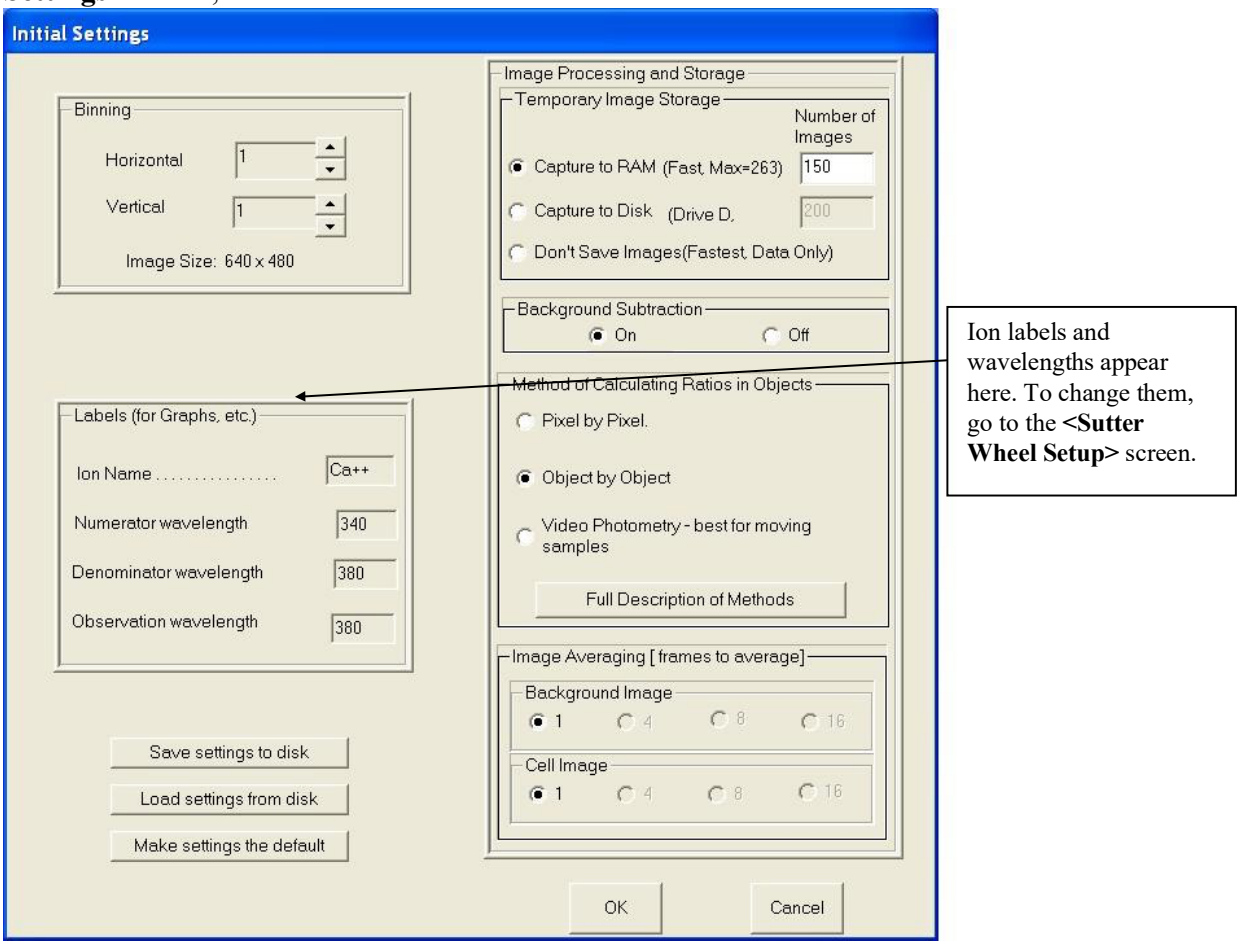
Enter the wavelengths of the filters in your wheel in the Position/Wavelength boxes. If there is a shutter in a particular position, enter the letters "shu", as above. Only the filters with numerical entries will be usable by the program. Position "0" on the wheel is not currently available, due to some Sutter software problems using this position at the fastest speed.

In the "Ion Label" box, enter the name of the ion you are measuring. **If you are conducting pH experiments, enter "pH" in this box (the system is then signaled to calculate ion levels with two decimals).** Select the numerator, denominator, and observation wavelengths for the experiment by clicking on the up/down arrows. The "observation wavelength" is any wavelength you might use to identify cells for analysis (like a 485 filter for identifying GFP markers). Only those positions with numerical entries will be available.

V. *InCyt Im2*™ PROGRAM: Setup > Sutter Wheel Setup

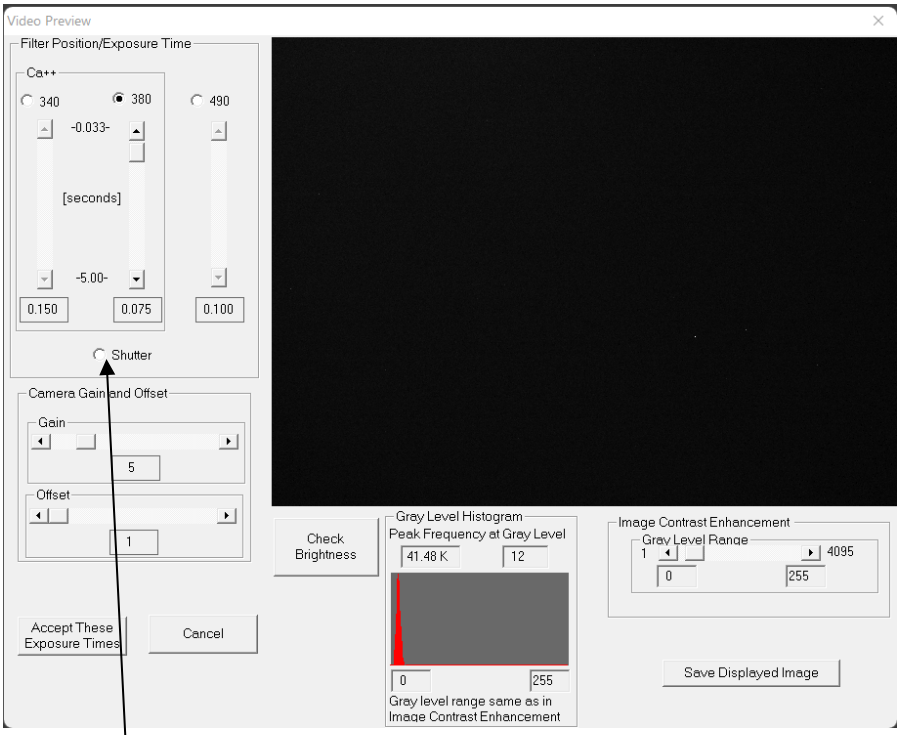
Hit the **<OK>** button when you have made the changes you desire. To exit without making changes, hit **<Cancel>.**

The ion and wavelength designations you have made will now appear in the **<Initial Settings>** screen, as shown below:



In **<Video Preview>,** and any other screen in which you see a live image, you will have the option of viewing your sample with the numerator wavelength, the denominator wavelength, or with the observation wavelength. For example, you might find the objects of interest in **<Video Preview>,** then you can look for those same objects in **<Capture Image>** in **< Experiment>** with the wavelength (i.e., to identify cells with GFP labeling). Here you will be given the opportunity to draw around the areas/cells of interest. The program will then collect data only from the areas that you indicated.

V. *InCyt Im2*™ PROGRAM: Setup > Sutter Wheel Setup



When you click on the **<Shutter>** radio button, the Filter Wheel is directed to find the shutter position closest to the numerator wavelength.

The Windows 10 program for the Sutter Wheel has two setup files: 1) The InCytIm2.SET or InCytIm1.SET found in our other programs, and 2) SUT_LAMDA_10_C.CFG. The SUT_LAMDA_10_C.CFG file contains the settings you designate in the **<Sutter Wheel Setup>** screen, as well as speed and switching time instructions for Wheel operations.

999	shu	340	380	shu	222	shu	shu	485	shu	**[position designations]**
485	**[observation wavelength]**									
33	**[default observation wavelength exposure time in milliseconds]**									
0	**[Sutter Wheel Speed -- from 0 (fastest) to 7 (slowest)]**									
60	100	135	175	210	**[switching times for moving 1, 2, 3, 4, and 5 positions]**					

The Sutter Wheel speed and corresponding switching times (last two lines of the file) have been optimized for your system. If you add or subtract filters from your wheel, you may need to change these parameters in this file (you can read it with Notepad). At this time Intracellular Imaging recommends using switching times that are at least 10ms longer than that specified by Sutter (see Sutter manual) for any given wheel speed, to ensure that the filters are in position when the measurement is being taken.

V. *InCyt Im2*™ PROGRAM: Setup > Sutter Wheel Setup

b. Manual Operation

There may be times when you may want to use the filter wheel without running the InCyt programs. Both the Sutter 10-2 and 10-C wheels allow you to change the filter position using the keypad.

With the Sutter 10-2, you must hit the **<LOCAL>** button on the keypad before you can utilize the keypad (this removes control from the CPU). With the Sutter 10-C, just press and release the number on the keypad that corresponds to the desired filter position (0-9).

Both filter wheels allow you to change the speed setting manually. PLEASE DO NOT CHANGE THE SPEED SETTING MANUALLY WHEN USING THE INCYT PROGRAMS. THIS WILL RESULT IN A LOSS OF CPU CONTROL OF SPEED, POSSIBLY RESULTING IN INCORRECT FLUORESCENCE MEASUREMENTS. If you change the speed settings manually, please turn the power to the Sutter Wheel controller off and back on to reset the settings before using the InCyt programs.

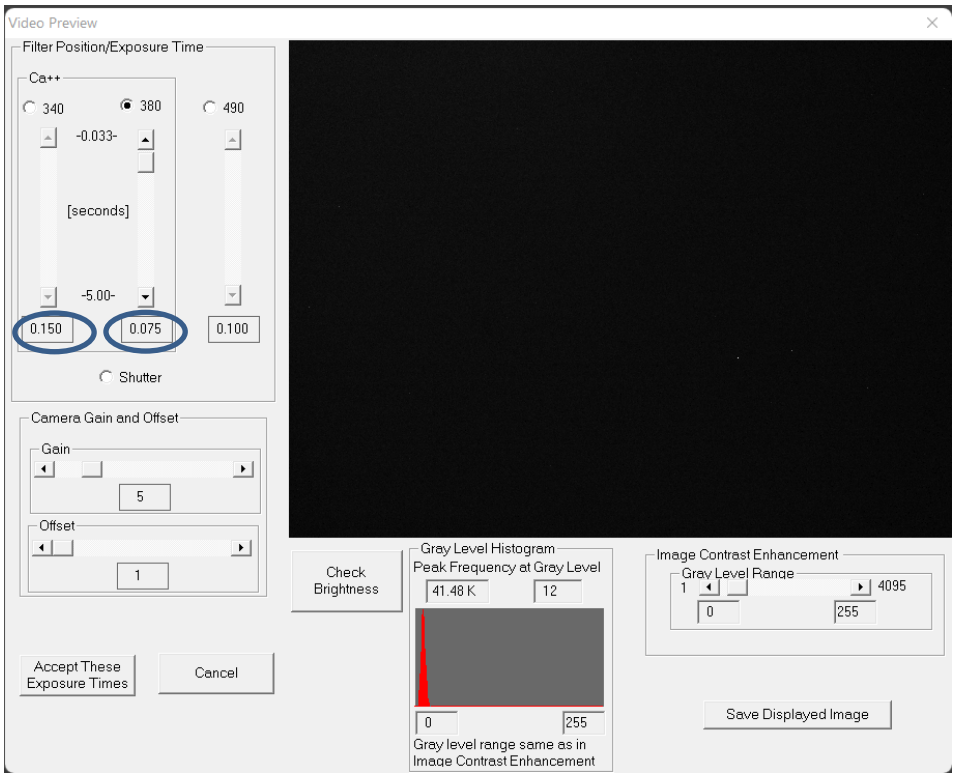
V. *InCyt Im2*™ PROGRAM: Video Preview

C. VIDEO PREVIEW

There is a detailed explanation of **<Video Preview>** in the Calibration section of this manual. You can enter **<Video Preview>** from many locations in the program, including the **<Main Menu>**. Any time you need to look at your cells through the camera, go to **<Video Preview>**. If you cannot see your cells properly with one wavelength, try another wavelength. The picture is refreshed at a rate determined by the exposure time. For example, as shown below, the 340nm picture will be refreshed about 6-7 times every second, while the 380nm picture will be refreshed 13 times a second. The various video previews are the only place in the program where you get a continuous "live" view of the sample.

> **IMPORTANT:** Resist the temptation to alter the relative exposure times after you have completed **<Calibration>**. Doing so without recalculating the calibration curve will result in erroneous measurement.

To demonstrate the impact of exposure times on the calibration curve, let's assume that a given cell has intracellular Ca^{2+} levels of 100nM and fluoresces with roughly equal brightness at 340nm exposed for 0.150 seconds and 380nm exposed for 0.075 seconds -- e.g., let's assume both gray-level readings are 100 -- for a ratio of 1.00:1.00. Now if you increase the 380 exposure time to 0.150 seconds (without comparably changing the 340 exposure) and take another reading, the gray level at 380 will have increased to 200 and the new ratio will be 0.5:1. If the calibration curve were built on 340/380 exposure times of 0.150/0.075 seconds, the system would now measure Ca^{2+} concentration as much lower than the actual 100nM. Therefore, if you change the exposure times, BOTH the 340 and the 380 times must be changed by the same percentage.

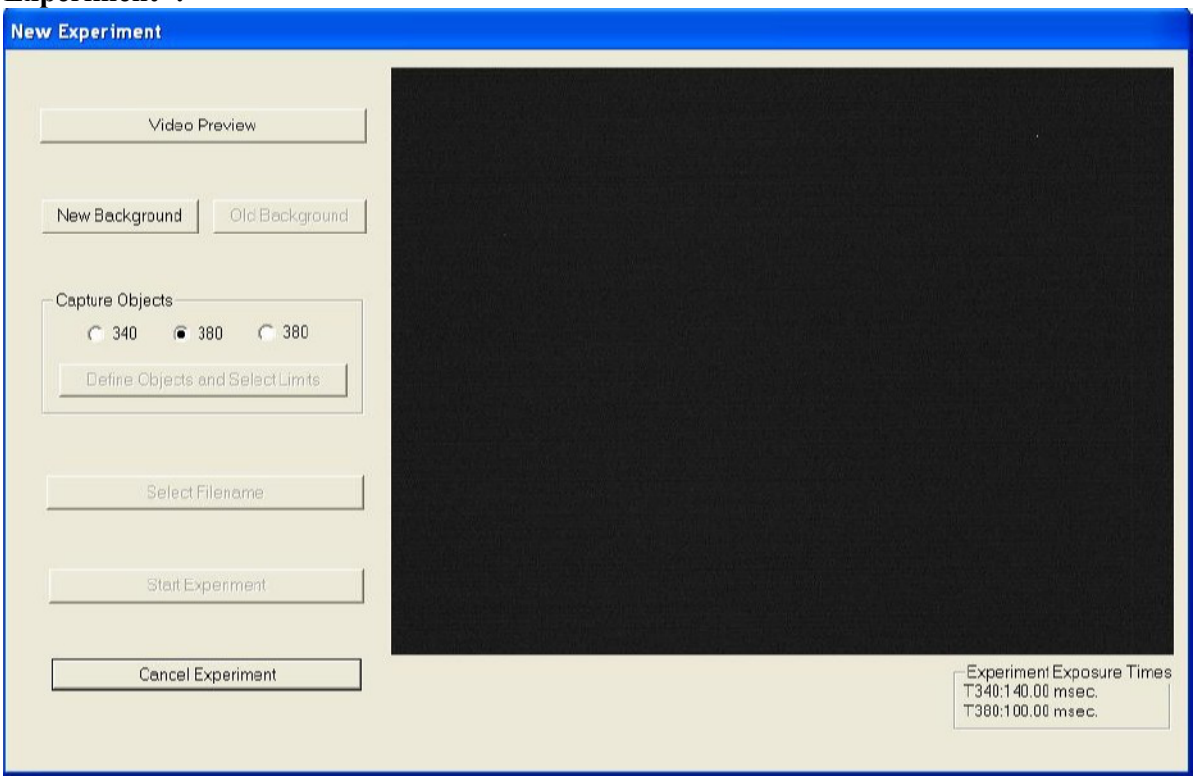


For example, you can alter **BOTH** exposure times by the same factor, so that if you have a plate of cells that loaded dye poorly, you have two choices. You may increase the lamp intensity, or you could choose to increase both exposure times from the current 0.150 second at 340nm & 0.075 second 380nm up to 0.450 second and 0.225 second, respectively. Note that both times have been increased by a factor of three.

V. *InCyt Im2*™ PROGRAM: New Experiment

D. NEW EXPERIMENT

At last we come to the section where you will actually conduct an experiment. From the top level menu, Click on <Experiment> and from the drop down menu select **<New Experiment>.**



Before starting the experiment, you will pre-select the cells or other areas of interest. After starting the experiment you will receive immediate pictorial and graphical tracking of cell responses. At the conclusion of the experiment, you will have an opportunity to save the images that were collected during the experiment. The graphical data are always saved automatically. Note that most menu items in the above dialog box are grayed out. As you complete each section, more of the options below that section will become active. Thus, the menu activates in a top down sequence. To begin with, only the <Video Preview> and the <New Background> options are available.

D.1. Video Preview

First go to <Video Preview> and select the field of cells you wish to use for the experiment. Adjust the lamp intensity and exposure times to give images of the cells that are well above background. Factors to take into consideration in choosing lamp intensity and exposure times include the following: (i) bright cells are better than dimmer ones; (ii) high lamp intensity allows shorter exposure times but promotes dye bleaching and photodamage to cells; (iii) longer exposure times increase cell brightness but slow down the rate of data acquisition. (For a review of the various features in <Video Preview>, see the Calibration section of this manual)

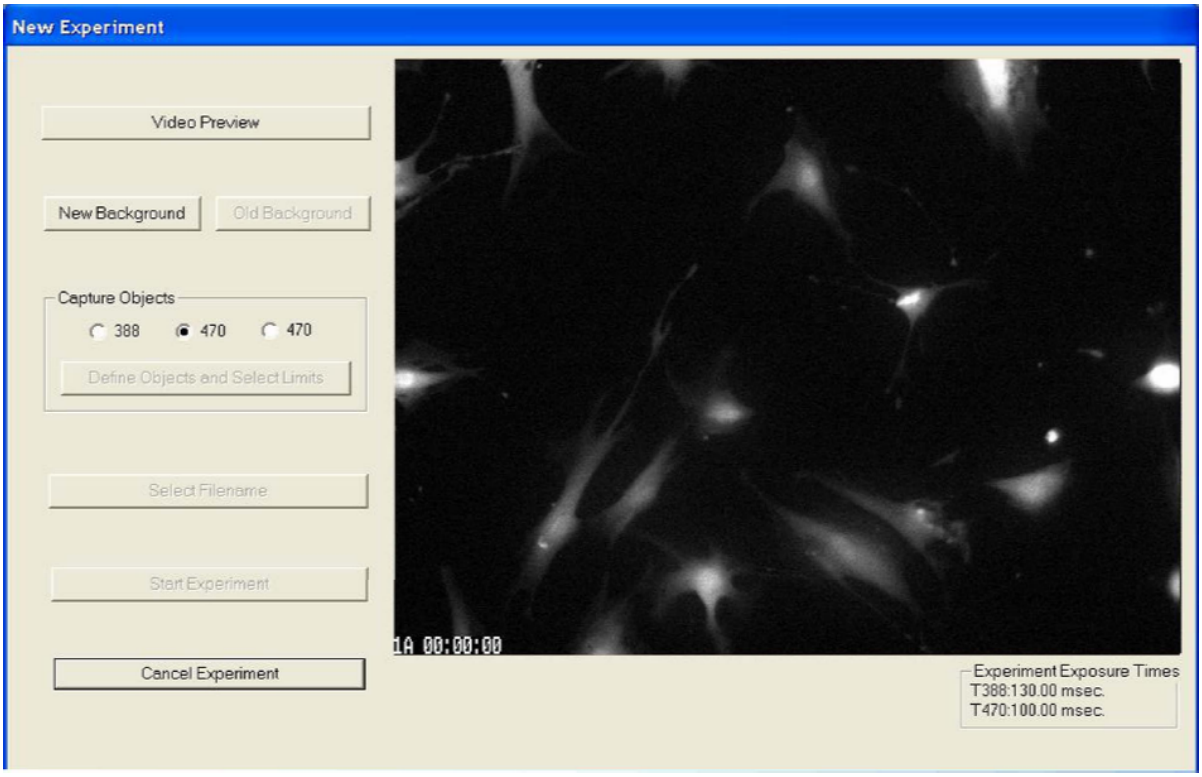
After you have selected the field of cells and adjusted the image brightness, click on <Accept these Exposure Times>.

D.2. Background Subtraction

Now it is time to get background images at both the 340 nm and 380 nm wavelengths. These backgrounds will be subtracted on a pixel by pixel basis from each cell image as it is collected during the experiment. (N.B. This assumes that in the <Setup> menu you have elected to have Background Subtraction turned on.) When you click on <New Background>, you will be asked to locate a background image. This can be done either by moving to an area of the dish that is devoid of cells or by defocusing the objective by lowering it all the way down. After you have done this, click on <OK> and background images will be captured at both wavelengths. The background image at the denominator wavelength (380 nm) will be displayed and you will be asked whether it is acceptable. This is done as a safeguard to make sure that the room lights are sufficiently dim and that you have not forgotten to remove the cells from the field of view. If the background image is reasonably dark, accept it.

If this is not the first experiment you have done in this session, then the <Old Background> bar will also be active. Using this option will avoid having to go through the process of acquiring a new background. Note, however, that you should not use this option if you have changed the lamp intensity.

D.3. Define Objects and Select Limits



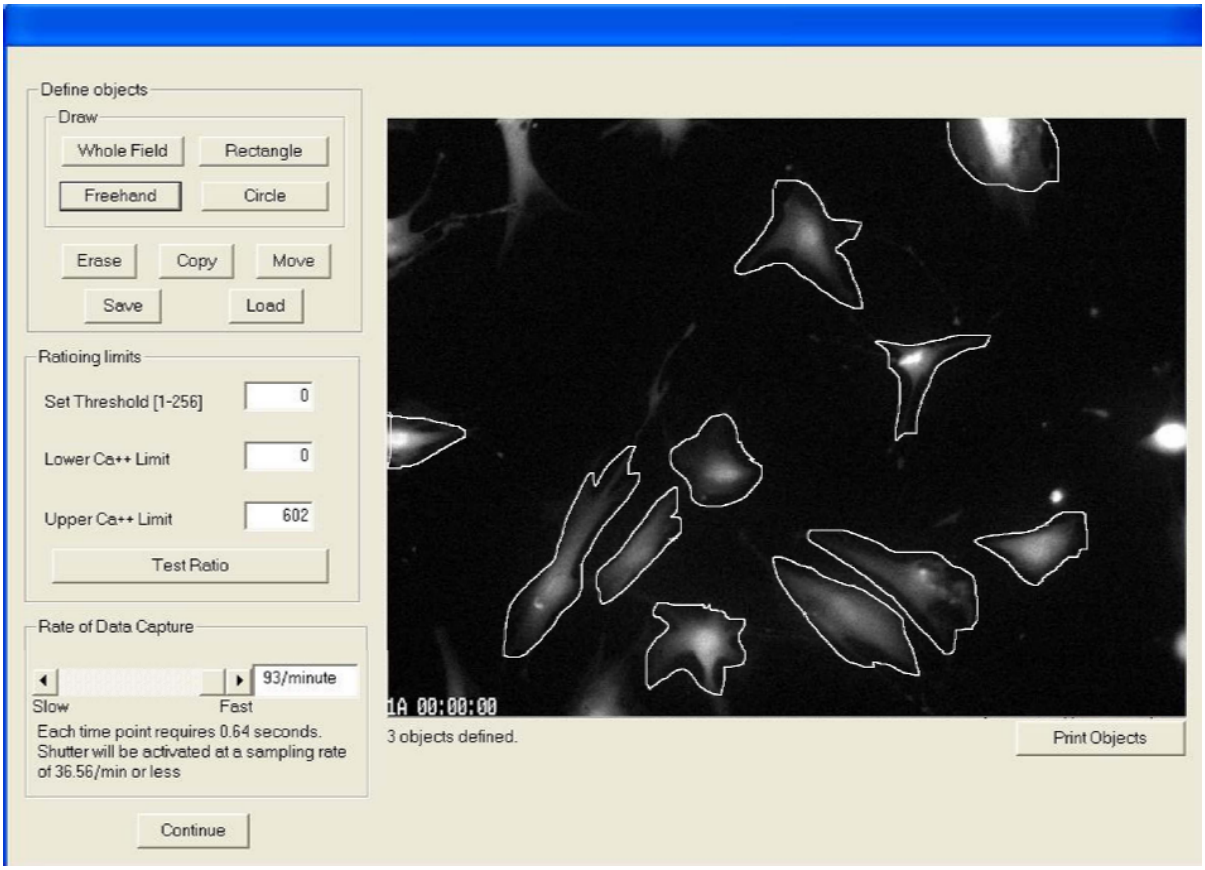
V. *InCyt Im2*™ PROGRAM: New Experiment

D.3. Define Objects and Select Limits (continued)

After having accepted a background, the <Capture Objects> box will become active. Select a wavelength from among the three radio buttons and re-acquire an image of the cells by either moving to an appropriate area of the dish or refocusing the objective, as the case may be. You are almost ready to click on the <Define Objects and Select Limits> bar. However, before you do this, it is important to understand that the particular wavelength that is currently selected by the radio buttons will determine which of two possible data groups the objects will be assigned.

If you select either of the wavelengths that are going to be ratioed (in the case of fura-2, the 340 nm or the 380 nm wavelengths), then the objects you define will be drawn in red and assigned to the class of fura-2 loaded cells. On the other hand, if you select the third (observation) wavelength, the objects you define will be drawn in green and will be graphed separately from the other objects during the experiment. For example, if you are working with cells that have been acutely transfected with GFP, it is likely that not all the cells will have been successfully transfected. You can first use the observation wavelength to identify the GFP positive cells and then go back to the 340 or 380 nm wavelength and use it to identify the remainder of the population.

After deciding on a wavelength, click on the <Define Objects and Select Limits> bar. The dialog box shown below will appear.



D.3. Define Objects and Select Limits (continued)

The top set of options, contained in a box labeled "Define Objects," consist of drawing and editing tools for defining the objects you wish to measure. After you have chosen one of the drawing methods, **use the LEFT mouse button to start the drawing operation in the viewing area. Use the RIGHT mouse button to stop the operation and exit the viewing area with the cursor.** You can select up to 100 areas of interest to measure. However, nesting of areas is not permitted, so that if you draw two objects which have even a single pixel in common, they will be treated as a single object.

<FREEHAND> This drawing tool is best for defining irregularly shaped objects**. A**fter you hit the **<Freehand>** button, your cursor becomes an arrow inside the picture. Hit the LEFT mouse button at the outer edge of the cell or cell area you wish to identify for measurement. Release the mouse button and trace an outline of the area of interest. When you are close to enclosing the area, hit the LEFT mouse button again and the figure will close. You have now created a measurement "mask" for this area. To start another mask, repeat the left-to-start and right-to-close sequence. To stop drawing, complete the figure you are working on and hit the RIGHT mouse button again. This will release the cursor from the constraint of operating within the borders of the image area.

To use the **<Rectangle>** drawing tool, start at one corner of the region you want to define then, with the left mouse button, click and drag to form a rectangle of the desired shape.

To use the **<Circle>** drawing tool, first left click on the center of the circle and then left click again to define a point on the perimeter.

Clicking on the **<Whole Field>** bar immediately draws a rectangle around the entire field of view.

<COPY> If you have many cells or cell areas of about the same size and shape that you wish to identify, the **<Copy>** function makes it easier. Rather than drawing each cell separately, draw one mask of a size appropriate for repeating and hit the RIGHT mouse button to exit the viewing area. Then hit **<Copy>** using the LEFT mouse button. This sends you back to the field of view. Move the cursor over the mask you wish to copy and hit the LEFT mouse button. With the cursor you can then drag a copy of this mask to a new location. When the mask copy is positioned properly, hit the LEFT mouse button again. Repeat by moving to a new location as many times as necessary. To stop copying, hit the RIGHT mouse button.

To use the **<Move>** tool, left click on the object you want to move, move the cursor to the new location, and left click again.

You can select up to 100 areas of interest to measure.

D.3. Define Objects and Select Limits (continued)

<ERASE> If you make a mistake in drawing the masks, hit the RIGHT mouse button to exit the viewing area. Then hit **<Erase>** using the LEFT mouse button. This sends you back to the field of view. Move the cursor anywhere on or within a mask, hit the LEFT mouse button -- and the mask disappears. Repeat for all masks you wish to eliminate or redraw. When finished erasing, hit the RIGHT mouse button.

<SAVE> If you wish to save the mask outlines that you have drawn, hit the **<Save>** button. The program will prompt you to provide a file name and the system will automatically append an **".OBJ"** extension ("OBJ" or "Objects") to the file name. (Note: temporarily saving the mask objects under the default filename can be useful, because you will not have to redraw the masks if for some reason you have to abort the experiment.)

<LOAD> This button allows you recall saved mask outlines. Hit the **<Load>** button and the system will prompt you to select a **".OBJ"** file. Select the correct file and the mask outlines will appear over the current field of view. If your cells have moved since you saved the objects, you may need to move the stage to realign the cells with the masks.

<PRINT MASKS> This button allows you to print out a copy of the mask shapes. You are also given the opportunity to print the number that the system will assign to each mask. If identifying the position and shape of each object with its calcium level is important, then it is essential that you print out the mask with the object numbers. This will be the only record you will have of shape and position after the experiment is over. Each cell's data is marked with the object number for that cell in the data file.

The **"Ratioing Limits"** box which lies immediately below the box with the drawing tools contains 4 items: Threshold, Lower Ca^{2+}, Upper Ca^{2+} and Test Ratio. These items are used to try to optimize the graph and the cell images that will appear during the course of the experiment. Of course you won't know for sure just what the best settings for these parameters are until after the experiment. But don't worry, they will either be optimized automatically later or you will have an opportunity to change them after the experiment.

<UPPER & LOWER Ca^{2+} LIMITS> Let's consider an example. If you were to set the upper Ca^{2+} limit at 600 nM and the Ca^{2+} in some of the cells actually went to 800 nM during the experiment, the graph line for those cells will go off scale during the experiment, but the data are not lost and when you go to the **<Graph Data>** option after the experiment is over, the system will autoscale the graph to 800 nM. Similarly, those cells whose Ca^{2+} rose to 800 nM will appear saturated in the ratioed images. However, by selecting the **<Ratio>** option from the top level menu after the experiment is over, you can re-ratio the cells using the higher, 800 nM value as your upper limit. We recommend keeping the Lower limit at zero with just a little practice, you will find that your initial guesses for these parameters are so good that post-experiment changes will not often be necessary.

D.3. Define Objects and Select Limits (continued)

<SET THRESHOLD> Thresholding allows the user to remove noise from measurements beyond that which was removed by subtracting the background. There are factors such as camera noise or very low levels of fluorescence in the cell preparation that have nothing to do with cell dynamics and are not appropriate to measure. Because these factors create pixels with very small gray levels, the ratios that they give rise to can appear as extremely high calcium concentrations. This "stray" fluorescence can be removed by the **<Set Threshold>** function. This function instructs the system to ignore a certain number of the lowest gray levels (up to 64). Any pixel inside the mask that has a gray level at or below the threshold in EITHER the 340nm or 380nm image is set to black (gray level 0) and will be ignored in determining calcium concentrations within that object.

While it is important to remove the noise, it is also important that the threshold is not set to high. If the threshold is too high, the ratioed image of cell Ca^{2+} may become substantially smaller than the actual cell size. To find a good value for the threshold, it is useful to draw the boundaries of at least one object so that they extend well beyond the actual area of the cell. These areas can be used to visually check for proper thresholding by setting the threshold just high enough so that these areas lose their "salt and pepper" appearance and become black, but not so high that you start to lose parts of the cells themselves.

<TEST RATIO> Clicking on this bar will cause the system to ratio the images within the object areas you have defined. This enables you to see the results of any changes you have made in the threshold or calcium limits.

<**RATE OF DATA CAPTURE>** This determines how often a measurement will be taken by the system. It is like a time lapse function. The fastest capture rate is calculated based on the exposure times set in **<Video Preview>**. You cannot take measurements any more frequently, but you can increase the time between exposures by typing in a larger number in the dialog box. If you select a capture rate less than the fastest, then the system will calculate the amount of time to be spent not involved in actually acquiring data, and if this is longer than 1 second, then the shutter will automatically be closed between data points in order to minimize photobleaching.

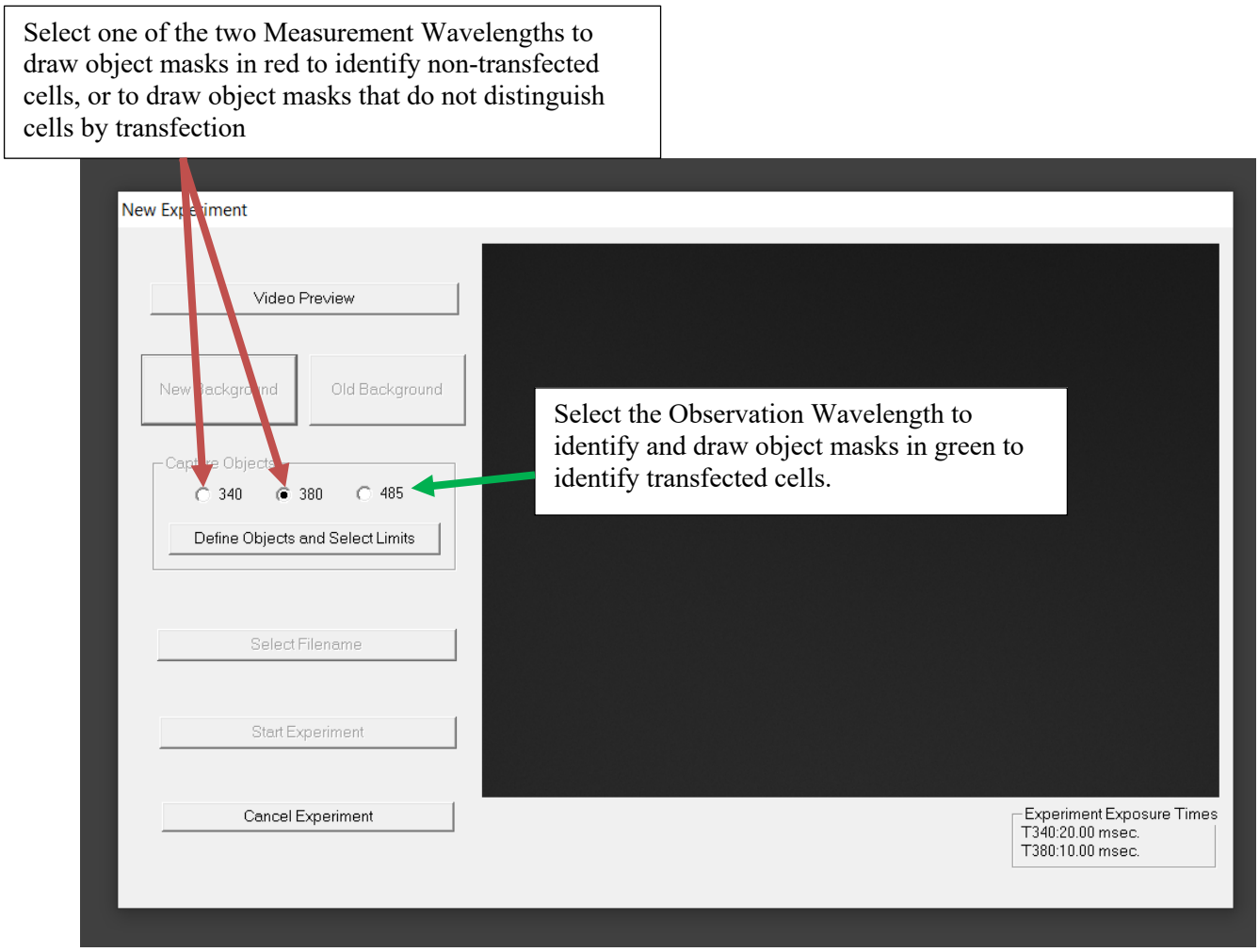
<CONTINUE> Once you are done defining objects and setting limits, click on **<Continue>** and you will be returned to the previous, "New Experiment" dialog box. You will still be able to return to this dialog box to define more objects or to adjust the limits.

V. *InCyt Im2*™ PROGRAM: New Experiment

D.4. Distinguishing Transfected Objects (Drawing Green vs. Red Objects)

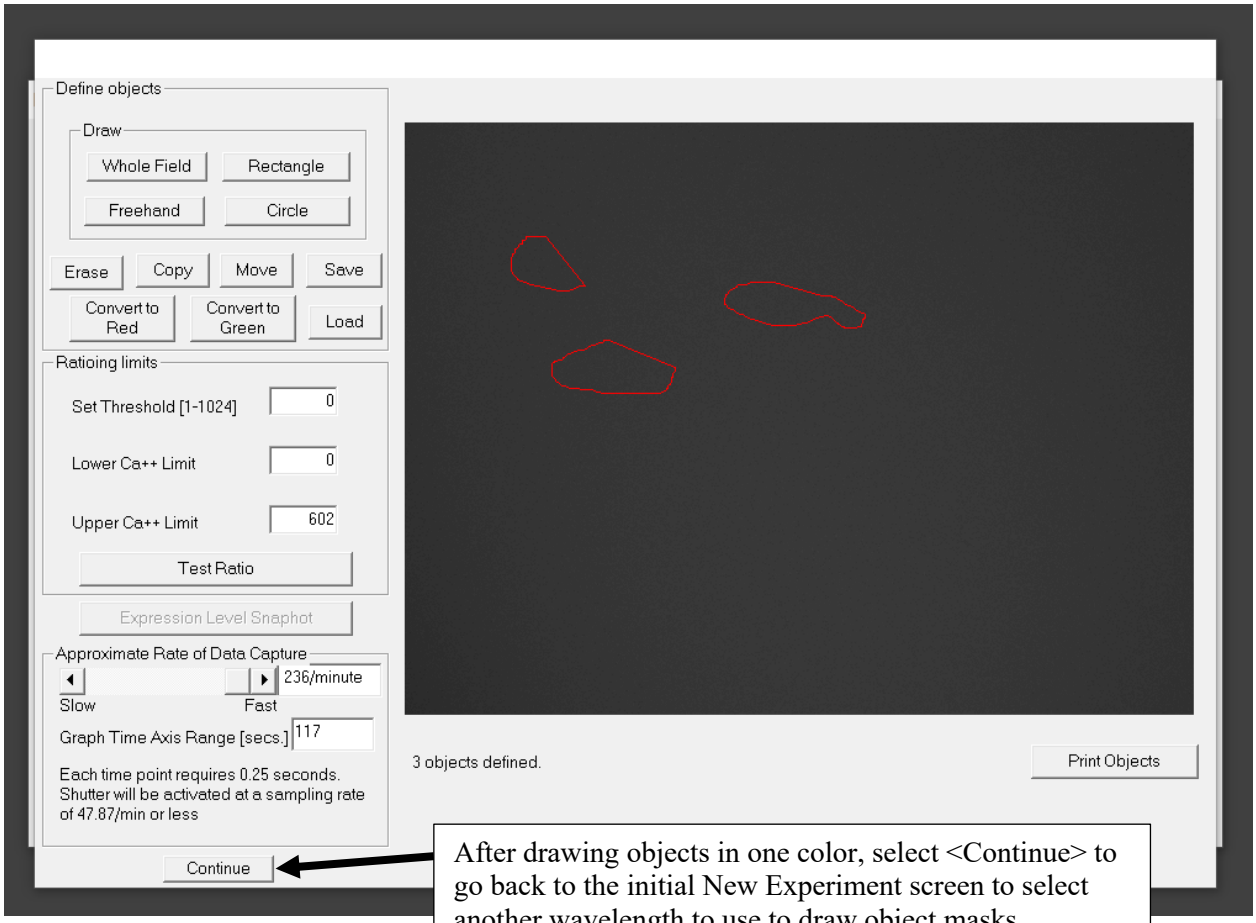
Newer versions of the program include an enhanced ability to identify different sets of objects during the experiment. This is most often used to identify cells which have been transfected with GFP vs. those which have not been altered. The program allows one to use a different color object mask for target cells, allowing one to more easily distinguish any difference in responses they may have during the experiment.

GFP (or any other transfection fluorophore) will be generally visible using the Observation Wavelength (485nm in the screen below). Therefore, if one chooses to <Define Objects and Select Limits> after selecting the Observation Wavelength, then the default color for the object masks will be green. If either of the Measurement Wavelengths are selected before one hits <Define Objects and Select Limits>, the default color for the object masks will be red.



V. *InCyt Im2*™ PROGRAM: New Experiment

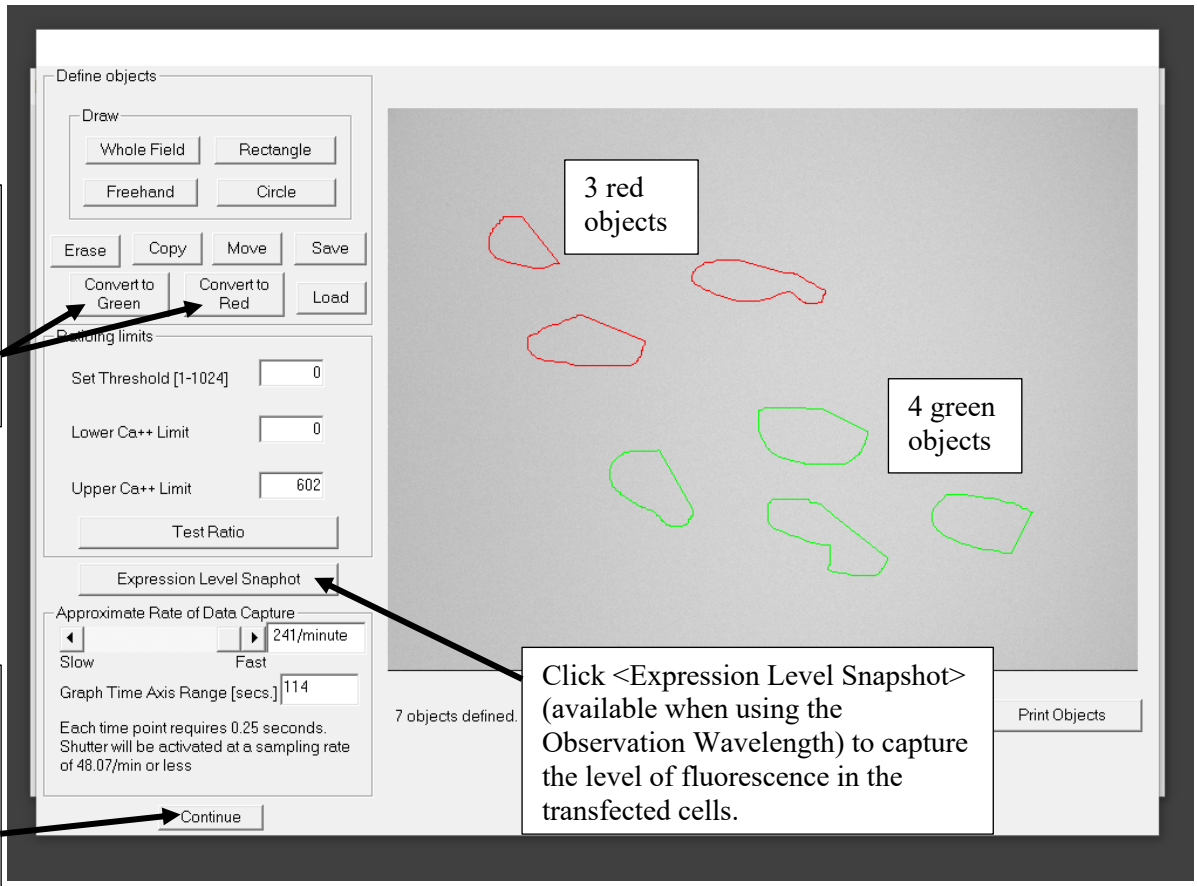
In the field below, there are red objects drawn after selecting 340 or 380 (one of the two Measurement Wavelengths).



After drawing objects in one color, select <Continue> to go back to the initial New Experiment screen to select another wavelength to use to draw object masks.

The screen on the next page shows the drawing of green object masks after the Observation Wavelength is chosen.

V. *InCyt Im2*™ PROGRAM: New Experiment



One can change the color of any object using these buttons.

Click <Continue> to move to the next step in the experiment.

Click <Expression Level Snapshot> (available when using the Observation Wavelength) to capture the level of fluorescence in the transfected cells.

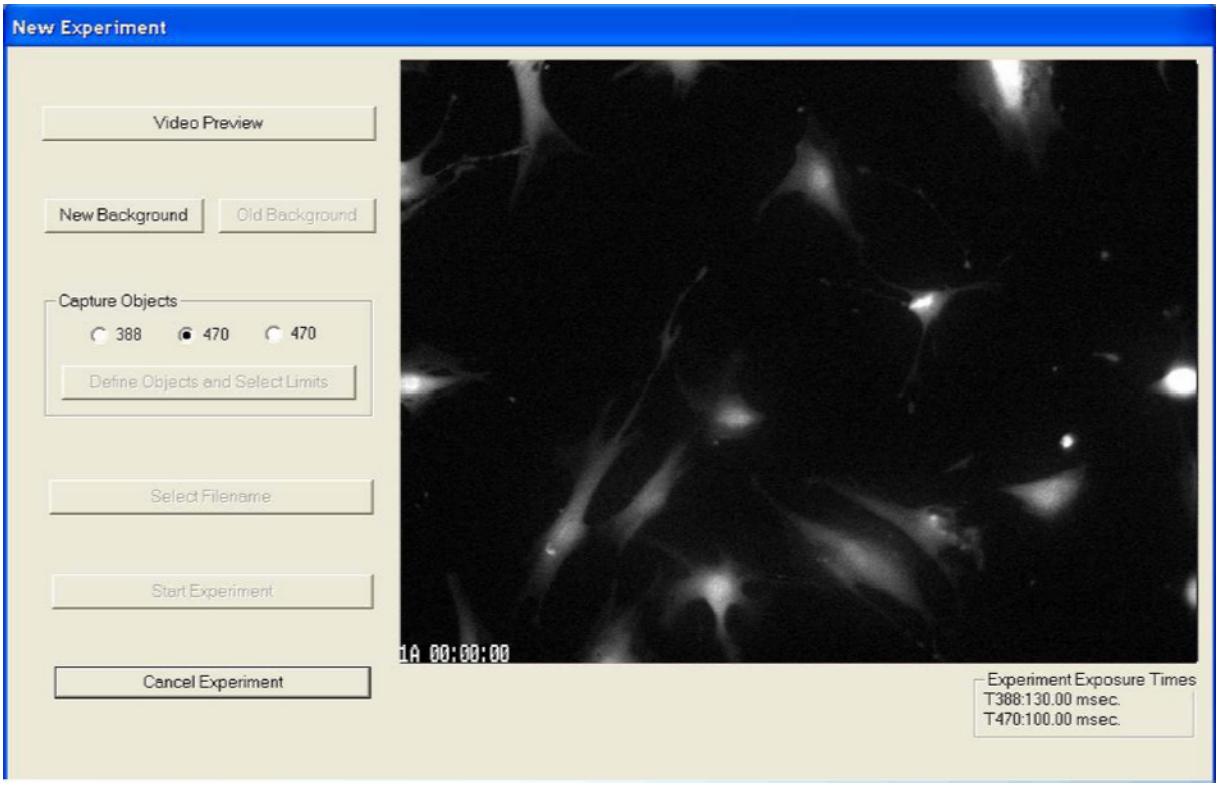
The Expression Level Snapshot file is a text file with the extension .ESS saved on the D:\ drive. It provides data on the wavelengths and exposure times used in the experiment, along with the total and average fluorescence of the objects (separated into Red and Green) using the Observation Wavelength. In an actual experiment, one would expect the green objects to show a much higher level of fluorescence than the Red Objects.

```
D:\InCytIm2\Demonstration ESS.ESS
Red_Objects=     3       Green_Objects=   4
Ion:                             Ca++
Wavelength_A:                    340
Wavelength_B:                    380
Wavelength_O:                    485
A_Exposure_Time:         20
B_Exposure_Time:         10
C_Exposure_Time:         45
DATA_AFTER_THIS_LINE
Object Number    Area    Total Fluorescence        Average Fluorescence
Red Objects
    1            1743         4801965                      2755
    2            2809         8247224                      2936
    3            3169         9063340                      2860
Green Objects
    4            3670        10958620                      2986
    5            2898         8514324                      2938
    6            3134         9116806                      2909
    7            3330         9956700                      2990
```

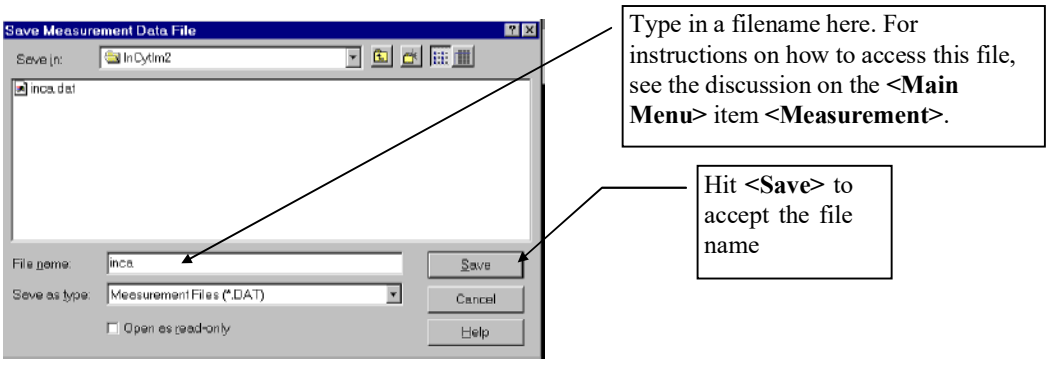
V. *InCyt Im2*™ PROGRAM: New Experiment

D.5. Select Filename

We now return to the "New Experiment" dialog box. The next step is to select the filename under which you wish to store both the measurement (.dat files)



<SELECT FILENAME> When you click on this bar, the dialog box shown below will appear. This file will be given a **".DAT"** extension.

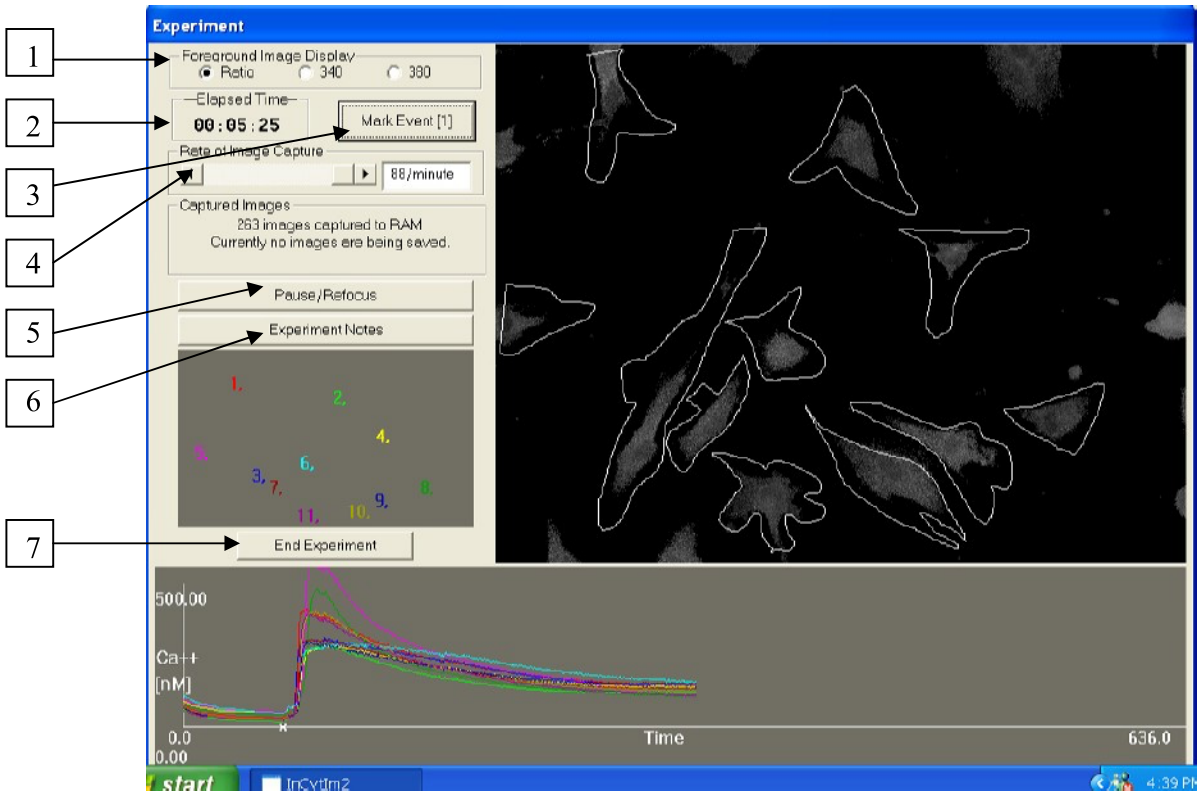


<START EXPERIMENT> Click on this bar to start the experiment. You will automatically be taken to the next dialog box.

V. *InCyt Im2*™ PROGRAM: New Experiment

D.6. Collecting Data

As soon as you have clicked on the <Start Experiment> bar, the following dialog box will appear.



The **"Experiment"** dialog box contains three **display regions**: the first (on the upper right) shows the images of the objects in the field of view; the second (on the left) shows the number and the color assigned to each object; the third (on the bottom) is a graphical display of the calcium value of each object. Both the images and the graph will be updated in real time as each new data point is collected.

1. **<FOREGROUND IMAGE DISPLAY>** These 3 radio buttons allow you to select which type of image will appear within the object masks that you have defined. You can toggle back and forth among any of them at any time. These images will be updated continuously during the experiment.

2. **<ELAPSED TIME>** Displays the time since the start of the experiment. You do not have any control over this, but you can use it to determine when to initiate an experimental manipulation.

V. *InCyt Im2*™ PROGRAM: New Experiment

3. **<MARK EVENT>** Clicking on this bar places an asterisk on the time axis of the graph and records the time of the event in the experiment data (.dat) file. This file can be viewed after the experiment is over. You can use the event marker as many times during an experiment as you want.

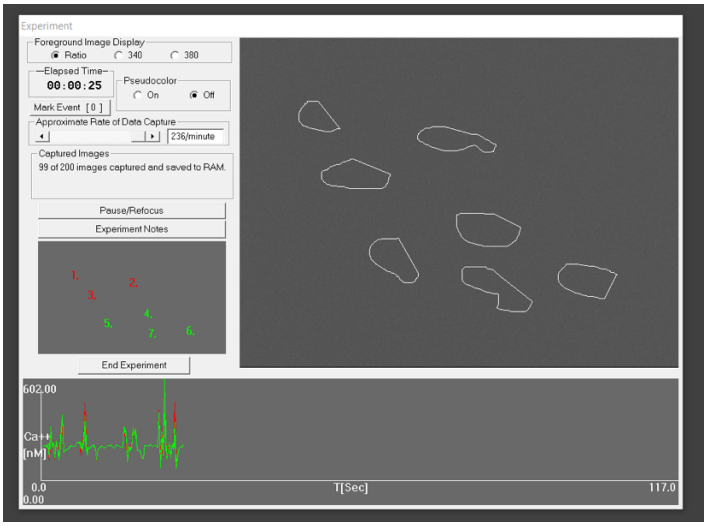
4. **<RATE OF IMAGE CAPTURE>** You can change the rate at which data points are captured during the experiment at any time using this slider bar. For example, you may wish to collect data slowly during the baseline period before an agonist is added, since calcium is not changing so there is no sense in using up a lot of the available images. Also, if you decrease the rate of image capture so that there will be more than one second of unused time between data points (i.e. when the camera is not capturing an image), the shutter will automatically close, thus decreasing dye bleaching and photodamage to the cells. (Note that when the rate of capture goes lower than 1/sec, the label on the slider will change from pts/min to pts/hour.)

5. **<PAUSE/REFOCUS>** If the cells move or go out of focus during the experiment, Ca^{2+} measurements will be incorrect. This button sends you to a new dialog box where you can refocus or reposition the cells inside the masks.

6. **<EXPERIMENT NOTES>** Clicking on this bar opens up the windows Notebook where you can record comments such as what was done when a particular event was marked. When you are finished writing a note for a particular event, just minimize the notebook so that you can open it up again to record your notes for the next event. When the experiment is over, save the notes with the same filename (but a different suffix) as the experiment.

7. **<END EXPERIMENT>** Clicking on this bar will end the experiment and take you immediately to a dialog box that gives you an opportunity to save some or all of the images.

If you have chosen to select red and green objects separately, they will be displayed in two different colors in the object map and the timeline. This allows one to immediately determine if there is a differentiation in response by the transfected cells.



D.7. Saving the Images

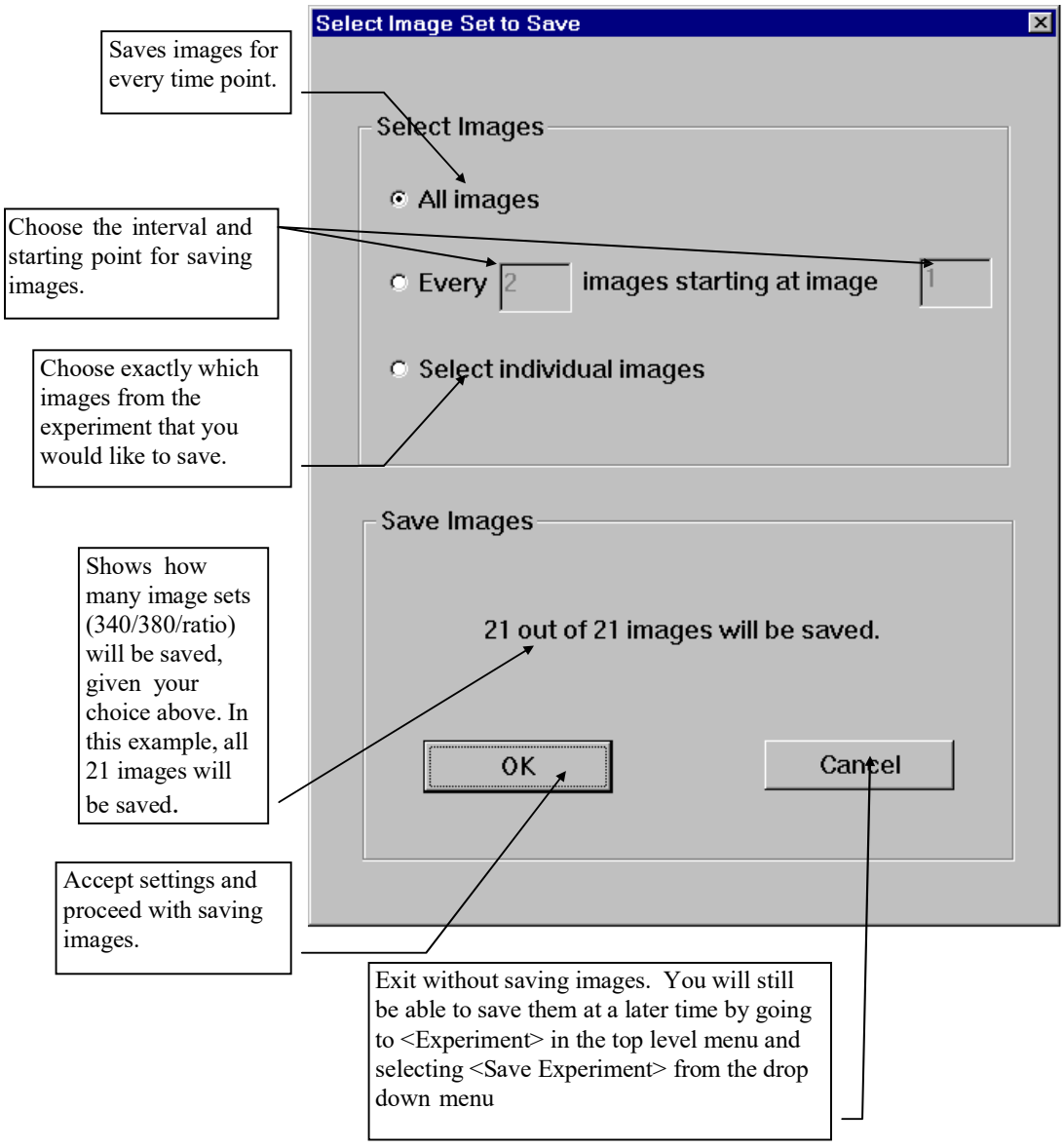
After you stop the experiment or the designated number of images has been captured, we **STRONGLY RECOMMEND** that you save the experiment immediately. You can cancel now and save the experiment later through the **<Experiment>** section of the **<Main Menu>**. However, because the images are currently being held in RAM or in volatile memory on the hard drive, the experiment data will be lost if the workstation is turned off or if another experiment is begun before saving.

NOTE: Whether or not you choose to save images, the data file containing the fluorescence intensities and calcium concentrations for each object at each time point have already been automatically saved. These data can be used to regenerate the graph that was created during the experiment by going to <Measurements> from the top level menu and selecting the <Graph Data> option from the drop down menu.

PLEASE NOTE: When you are saving images, you will have the ability to save the two Measurement Wavelength images, the Ratio Images, and an Observation Wavelength image. If you have elected to draw red and green object masks to differentiate transfected cells, it is HIGHLY RECOMMENDED that you save the Observation Wavelength image as an additional record of your experiment.

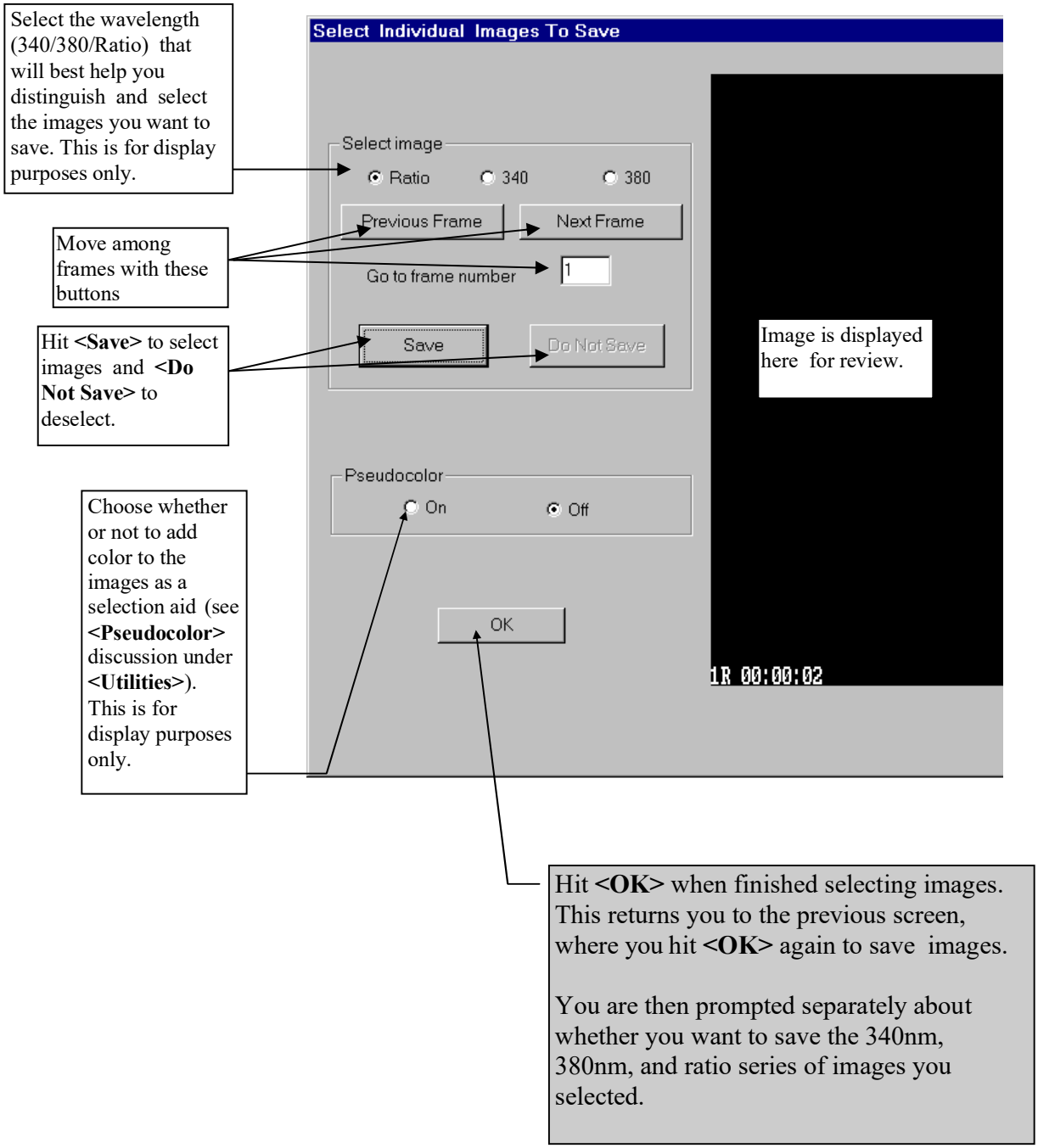
V. *InCyt Im2*™ PROGRAM: New Experiment

The first step is deciding which time points you want to save images. Because each unbinned image contains about 2/3 of a Mbyte of data, saving all the images from an experiment can consume a very large amount of disc space. For example, each time point consists of a 340 nm, a 380 nm and a ratioed image for a total of 2Mbytes, so that if you have saved images for 250 time points, saving them all to permanent memory will use up ½ Gbyte of disc space! For this reason, the program provides you with the opportunity to save all, some or none of the images.



V. *InCyt Im2*™ PROGRAM: New Experiment

If you choose the **<Select Individual Images>** option above, this screen will help you choose.



Hit **<OK>** when finished selecting images. This returns you to the previous screen, where you hit **<OK>** again to save images.

You are then prompted separately about whether you want to save the 340nm, 380nm, and ratio series of images you selected.

V. *InCyt Im2*™ PROGRAM: Re-Ratio Images

E. RE-RATIO IMAGES

1. When to use this function

You can re-ratio an experiment's images at any time, including immediately after having done the experiment. Two examples of when you might wish to do this would be:

(i) When setting up to do the experiment your choice of the upper Ca^{2+} limit was too low thus giving rise to ratioed images that became saturated.

(ii) You did not set the Threshold for measurement high enough, resulting in a lot of noise in non-cell regions of the image.

2. The sequence of steps

Step 1: Load the experiment you want to re-ratio. If you have just finished an experiment, the images are already loaded, even if you haven't saved them yet. The program will need the 340nm and 380nm images to work with. You may want to first review the old ratioed images to compare the changes that you make.

Step 2: Choose **<Ratio Images>** from the **<Main Menu>**.

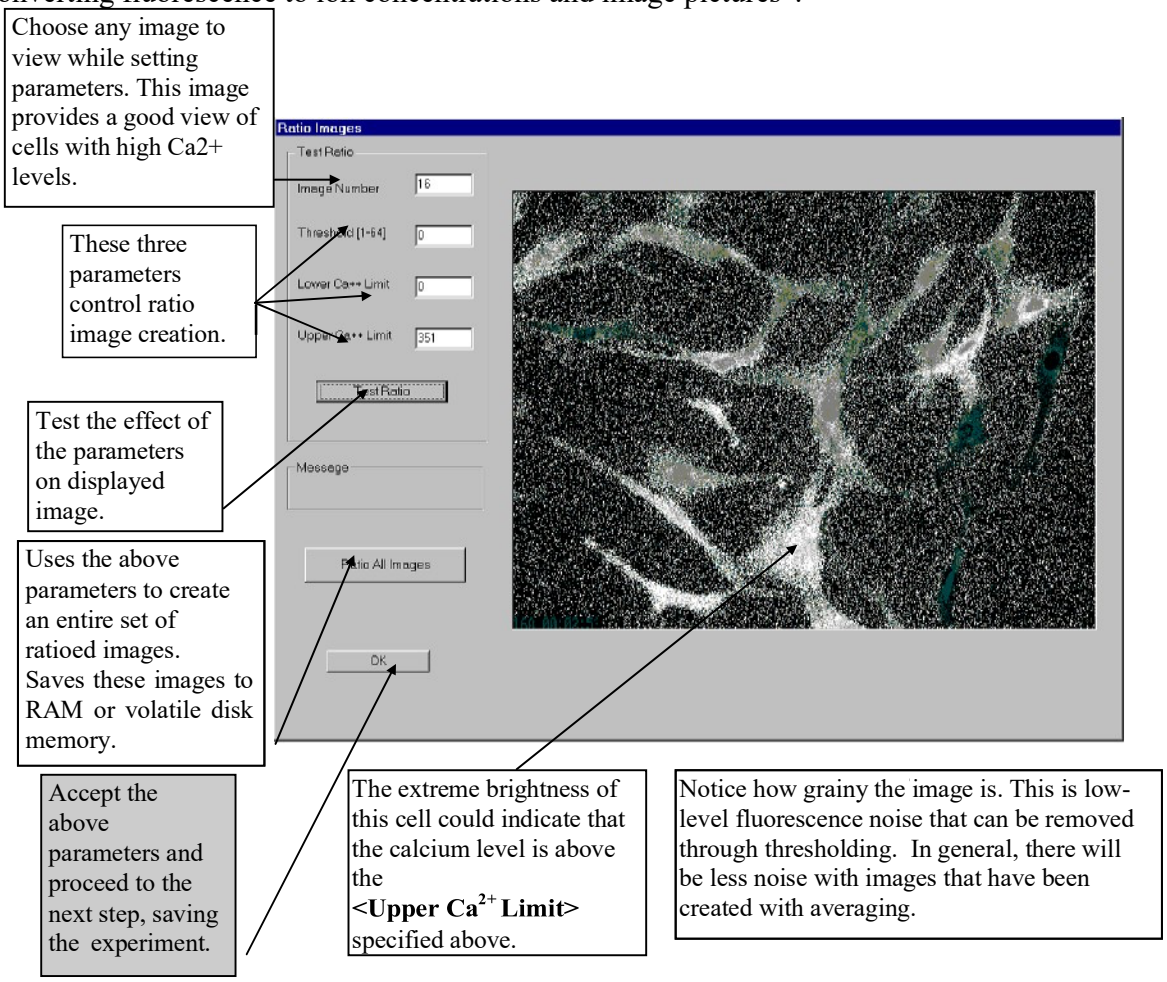
Step 3: Ratio images as described under heading "3. Creating Ratioed Images" below. When you find the correct parameters, be sure to hit the **<Ratio All Images>** button.

Step 4: Resave the experiment using **<Save Current Experiment>** from the **<Experiment>** menu as described in Section V.C.4. There is no need to resave the 340nm and 380nm images, but you must save the new ratio images. Again, you may save the experiment under a different name if you want to keep both the old and the new ratio images.

V. *InCyt Im2*™ PROGRAM: Re-Ratio Images

3. Creating Ratioed Images

After the experiment is stopped or completed, the system will prompt the user to set parameters for creating images that are the ratio of the 340nm and 380nm image. The process for converting ratio figures to an image is discussed in section V.A.3.d., "Converting fluorescence to ion concentrations and image pictures".



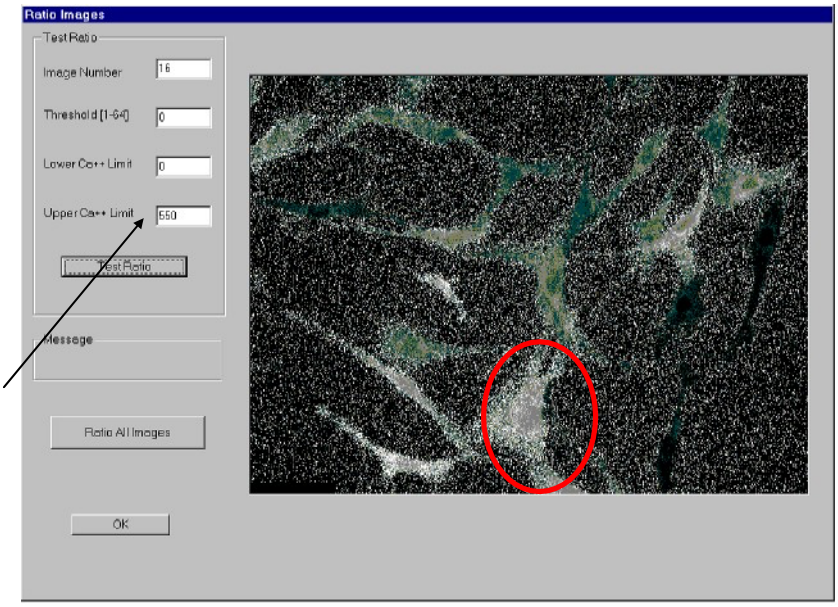
<Lower Ca++ Limit>
<Upper Ca++ Limit>

These limits affect the creation of ratioed images only, and DO NOT affect calcium measurements. These parameters determine how the program distributes the 256 gray levels across the Ca^{2+} levels. Any calcium concentration below the **<Lower Ca++ Limit>** will be set to black (gray level 0); any calcium concentration above the **<Upper Ca++ Limit>** will be set to bright white (gray level 255). We recommend keeping the Lower limit at zero and setting the Upper limit just higher than the maximum Ca2+ level you observe during the experiment (the high-calcium level can be determined later and the images can be re-ratioed, if necessary). The image below shows the impact of setting a higher **<Upper Ca++ Limit>** on the cell at the bottom of the screen.

V. *InCyt Im2*™ PROGRAM: Re-Ratio Images

Increasing the Upper Limit from 351nM to 550nM dims the cell at the bottom of the screen and provides more discrimination at high calcium levels.

However, it does not affect the noise level in the image.



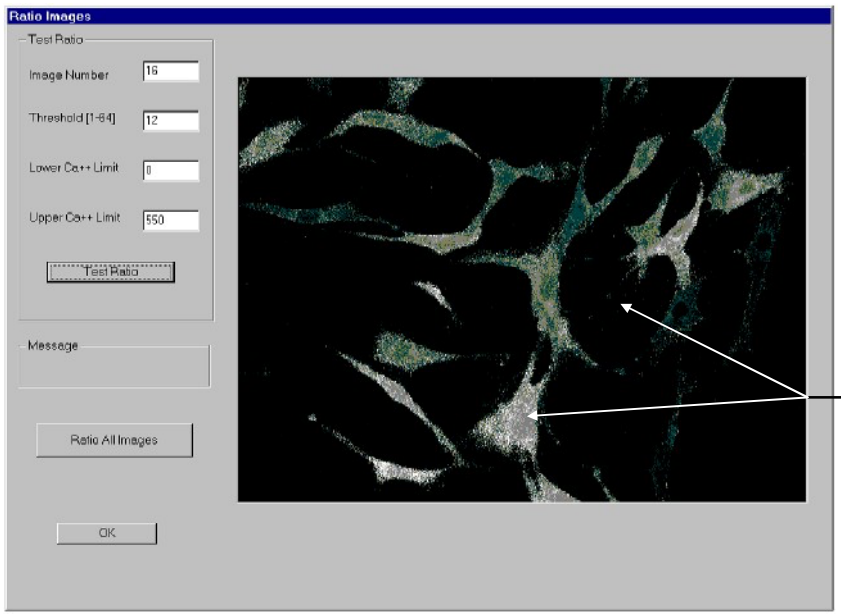
<Threshold (1-64)>

Thresholding allows the user to remove noise from measurements beyond that which was removed by subtracting the background. There are very low levels of fluorescence in the cell preparation that have nothing to do with cell dynamics and are not appropriate to measure. This "stray" fluorescence can be removed by this parameter. This function instructs the system to ignore a certain number of the lowest gray levels (up to 64). Any pixel in the image that has a gray level at or below the threshold in EITHER the 340nm or 380nm image is set to black and will be ignored.

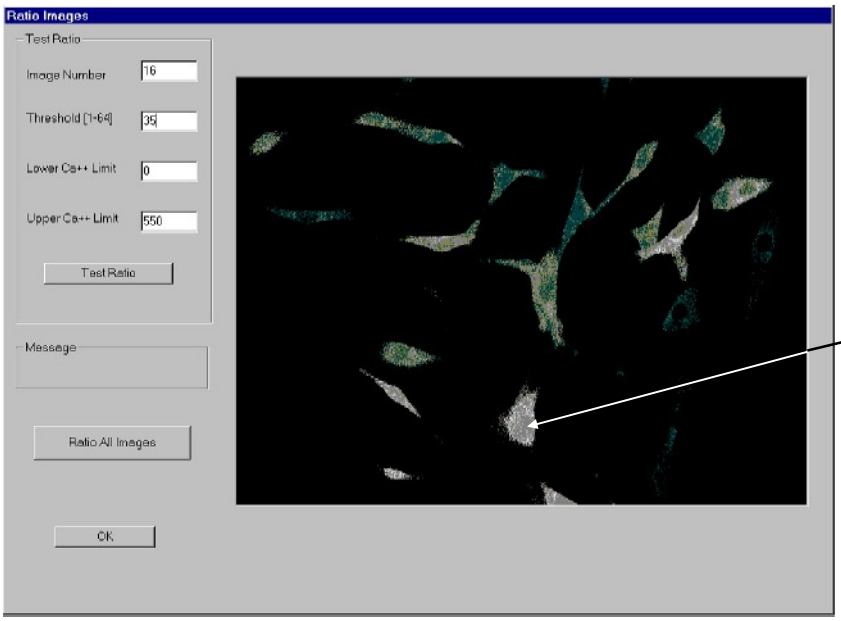
Unlike the Upper and Lower calcium limit parameters, THE THRESHOLD DOES AFFECT CALCIUM CONCENTRATION MEASUREMENTS. Only fluorescence above this threshold will be displayed and used in measuring ion concentration levels.

Therefore, while it is important to remove the noise, it is also important that the threshold is not set too high. If the threshold is too high, Ca^{2+} measurements will be understated. This is because as Ca^{2+} levels rise to high levels, the 380nm image dims. If the threshold is set too high, these low 380nm responses (high Ca^{2+} responses) will be eliminated from the analysis. Set the threshold just high enough so that the area between the cells is mostly black, but not so high that you start to lose parts of the cells themselves. The images below show the effect of increasing the threshold on the image.

V. *InCyt Im2*™ PROGRAM: Re-Ratio Images



Here a threshold of 12 produces a nice dark background and clear, complete cell images. This is a good image.



However, at a threshold of 35, cell images are clearly diminished and information is lost.

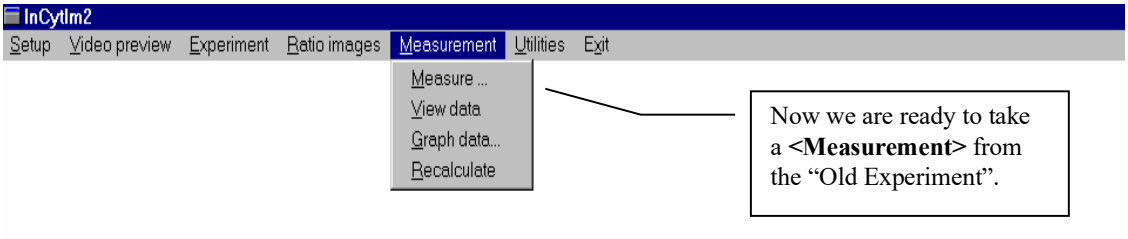
The threshold of 35 was chosen here to dramatically demonstrate the effect of an improper ratio. In this experiment, a threshold of 20 would also have been too high.

When you feel you have the right parameter levels, hit the **<Ratio All Images>** button to create a full set of ratioed images

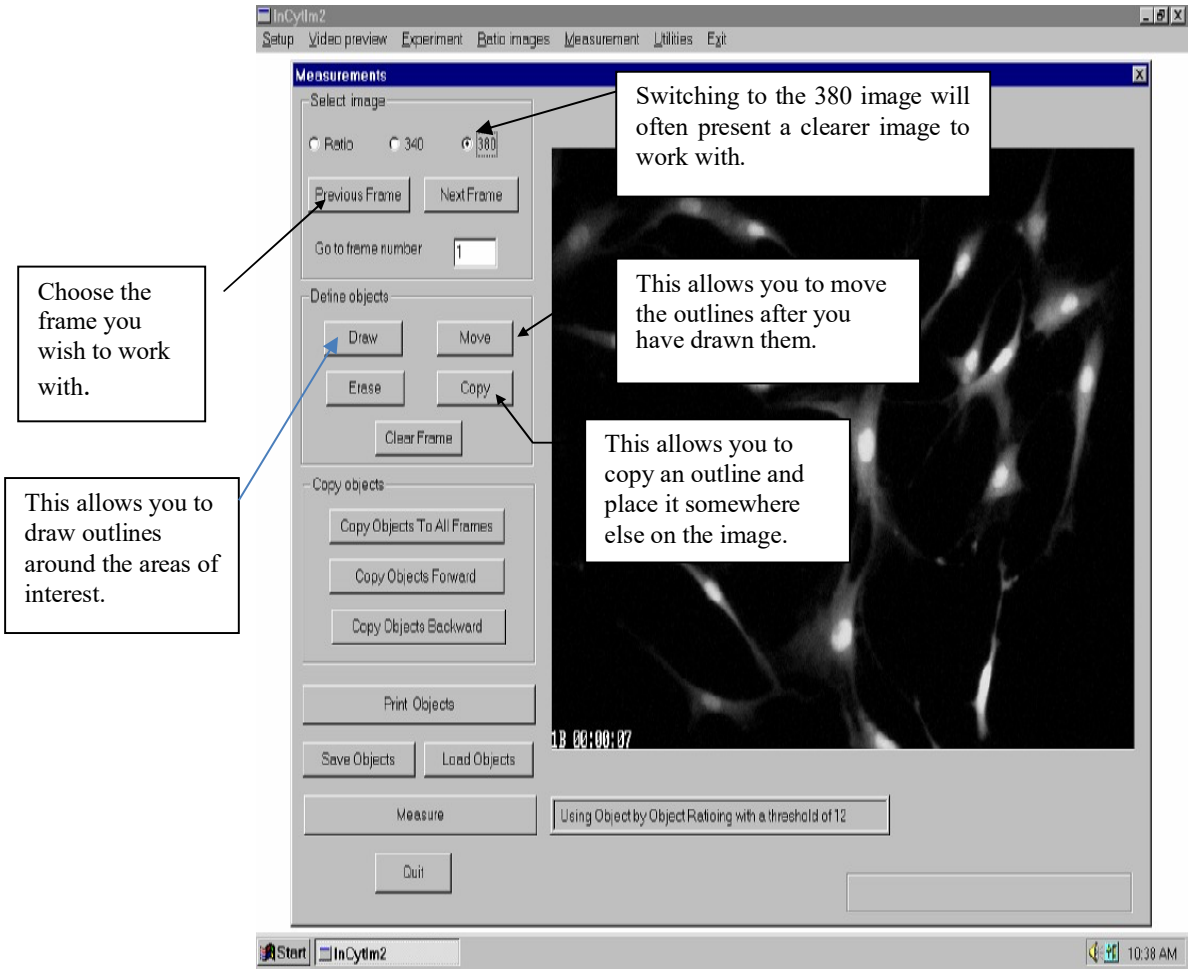
V. *InCyt Im2*™ PROGRAM: Measurement > Measure

F. MEASURING THE DATA

1. Measurement



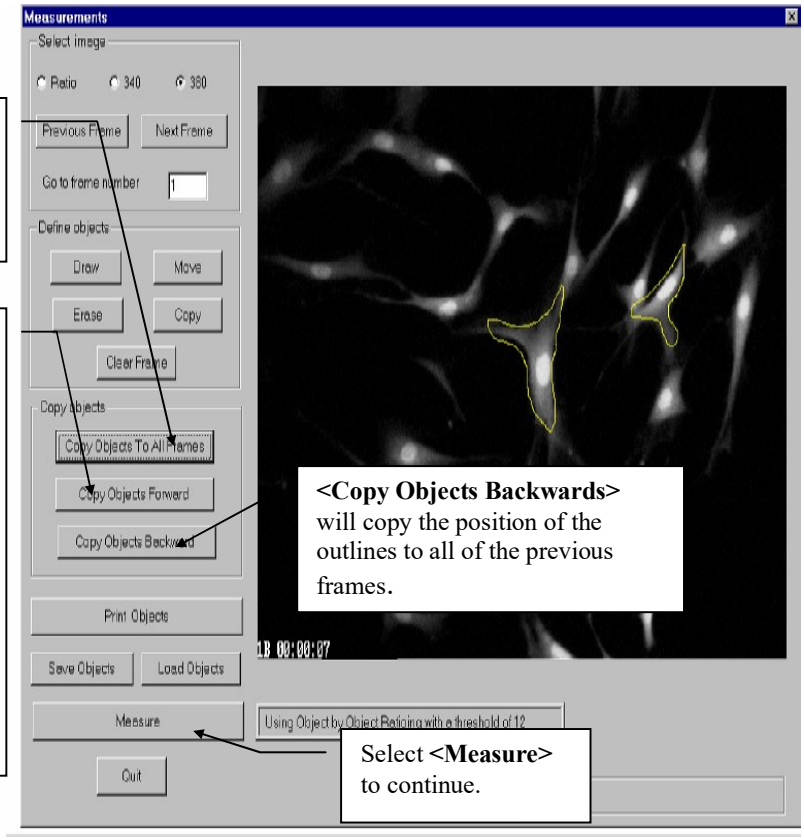
This screen, **<Measure>**, allows you to select the objects that you wish to take measurements from. You can draw outlines around the cells that you wish to collect data from. You can then manipulate those outlines so that they are copied to all of the frames of the experiment. This screen also gives you the ability to correct the placement of the object outlines if the sample moved at some point during the experiment.



V. *InCyt Im2*™ PROGRAM: Measurement > Measure

After you trace the images, remember to copy the objects to all of the frames.

If the cells move at some point during the experiment, it is still possible to get an accurate measurement. Go to the frame where the cells moved, click on **<Move>** to move the outlines to the new position, and then click on **<Copy Images Forward>**. This will change the position of the outlines for all of the remaining frames.

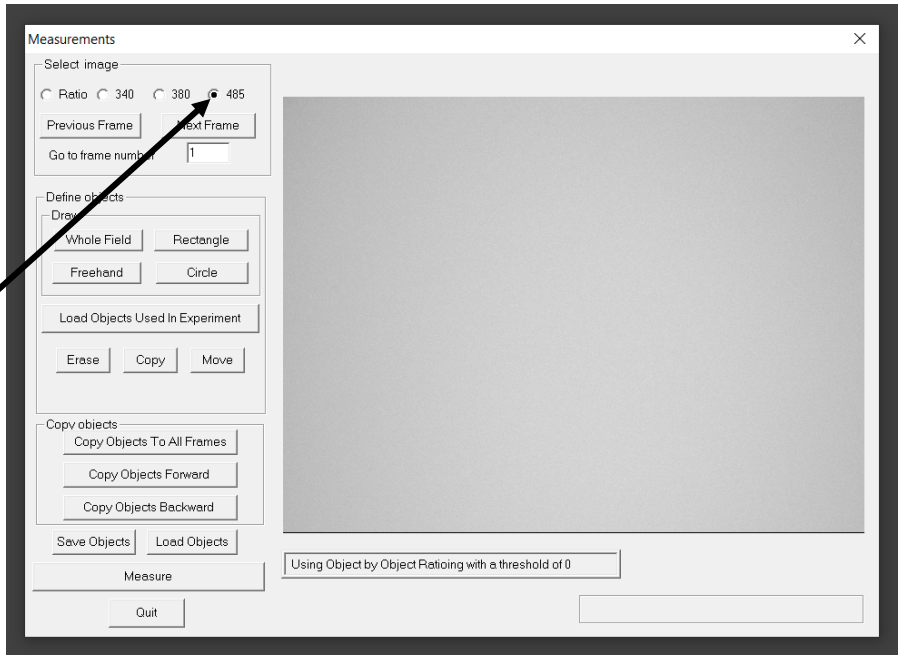


<Copy Objects Backwards> will copy the position of the outlines to all of the previous frames.

Select **<Measure>** to continue.

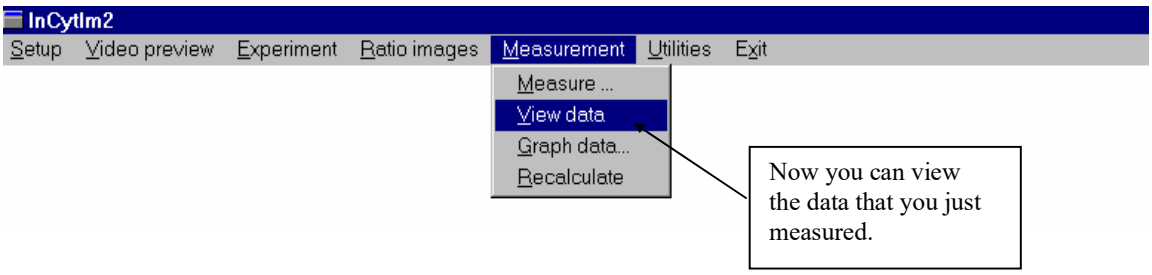
If you elected to save the Observation Wavelength, this image will be available to you whenever you remeasure your experiment.

The Observation Wavelength will also be available to draw new object masks if that image was saved after the experiment.



V. *InCyt Im2*™ PROGRAM: Measurement > View Data

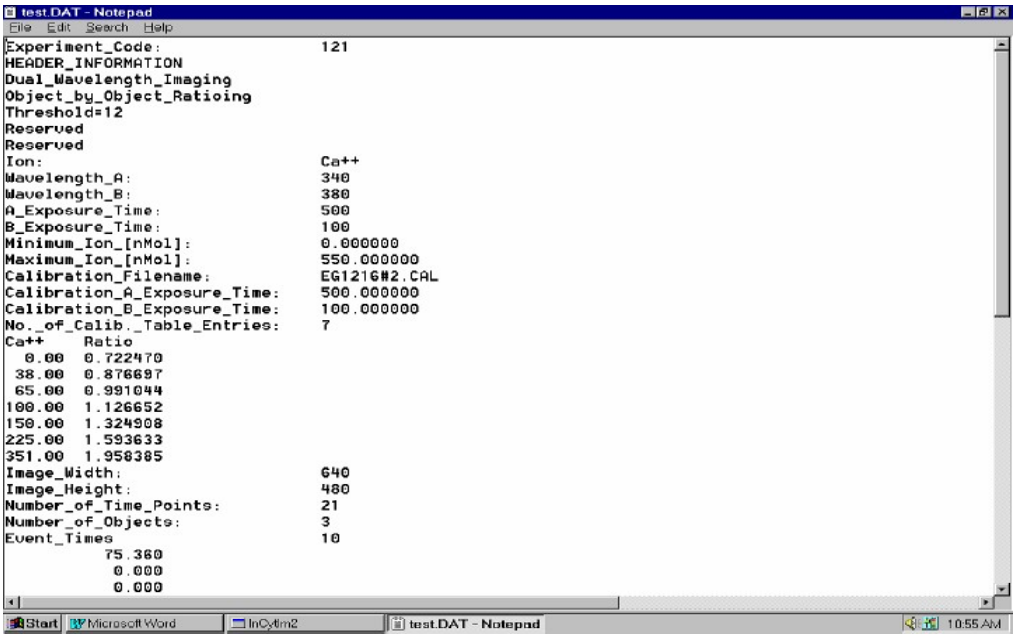
2. View Data



The data will be presented in notepad form. The file that the data is saved in is a tab-delimited ASCII file, which can easily be transferred to almost any spreadsheet program. The data that is presented in the heading includes the ion being measured, the wavelengths it is being observed at, and the exposure time at each wavelength. It also includes the minimum and maximum ion measurements, which can be set under **<Ratio Images>**. The calibration information is then listed, including the calibration table which can be set up under **<Setup>**. It also lists the size of the image, the number of images taken during the experiment, and the number of objects you outlined. After scrolling down to the bottom of the heading section, you will find the actual data from the experiment. The data is arranged in columns so that you can see the fluorescence of 340 and 380 wavelength light that is measured at each time point and the calculated amount of calcium at the time point. This table includes the data from all of the cells that you outlined. The number in parenthesis indicates which cell the data corresponds to.

V. *InCyt Im2*™ PROGRAM: Measurement > View Data

a. Header

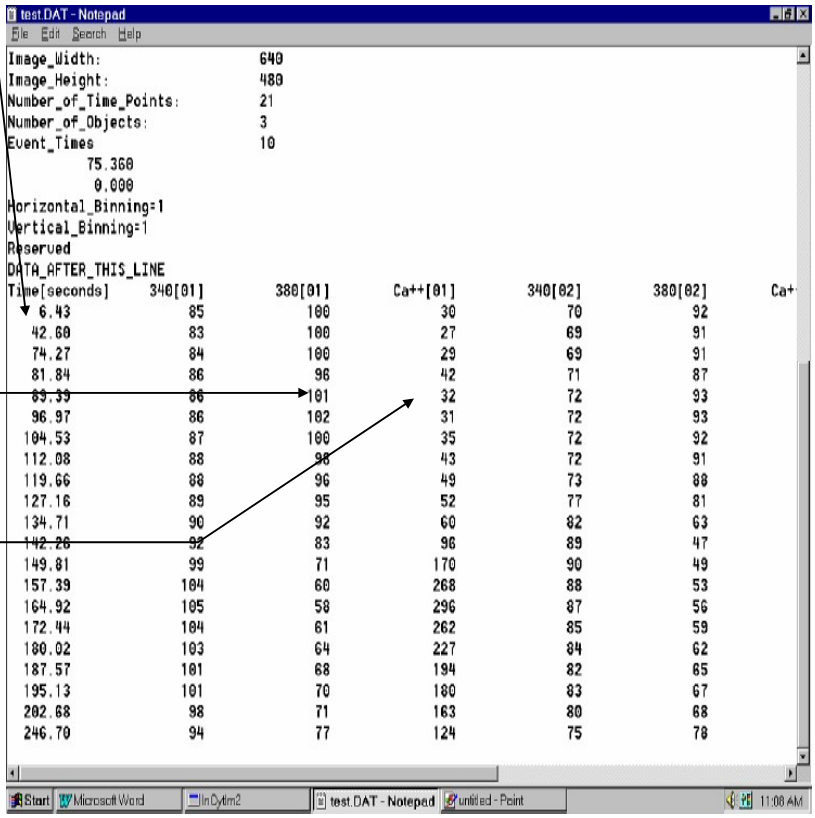


b. Experimental Data

The times at which measurements were taken.

The fluorescence level of 380 wavelength for the 1st object at each measurement.

The calculated calcium level for the 1st object at each measurement.



V. *InCyt Im2*™ PROGRAM: Measurement > View Data

If you selected red and green object masks in your experiment, they will be noted in the experiment data file:

```
Experiment_Code:                127
HEADER_INFORMATION
Dual_Wavelength_Imaging_With_Two_Object_Classes
Object_by_Object_Ratioing
Threshold=0
Red_Objects=    3       Green_Objects=  4
Reserved
Ion:                            Ca++
Wavelength_A:                   340
Wavelength_B:                   380
A_Exposure_Time:                20
B_Exposure_Time:                10
Minimum_Ion_[nMol]:             0.000000
Maximum_Ion_[nMol]:             602.000000
Calibration_Filename:           EGRS081721#1_20x.CAL
Calibration_A_Exposure_Time:    150.000000
Calibration_B_Exposure_Time:    75.000000
No._of_Calib._Table_Entries:    6
Ca++    Ratio
  0.00  0.640512
 38.00  0.888938
100.00  1.317618
225.00  2.135508
351.00  2.988854
602.00  4.169647
Image_Width:                    640
Image_Height:                   480
Number_of_Time_Points:          195
Number_of_Objects:              7
Event_Times                     0
Horizontal_Binning=1
Vertical_Binning=1
Reserved
DATA_AFTER_THIS_LINE
Time[seconds]     340[01]         380[01]         Ca++[01]                340[02]         380[02]
    0.2600                  1382            687             206                     1469            731
    0.5100                  1374            686             204                     1464            731
    0.7600                  1332            644             214                     1427            697
    1.0100                  1250            644             195                     1352            684
    1.2600                   597            304             198                      679            333
    1.5100                   722            503             118                      742            496
    1.7800                  1006            392             288                     1071            407
```

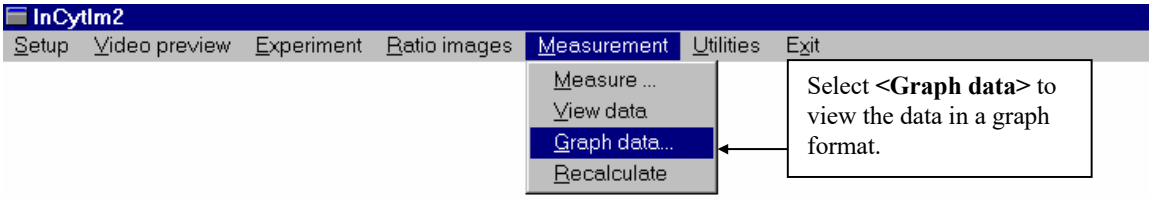
The numbers of red vs. green objects are noted here.

Red objects are listed /numbered before the green objects. Here [01], [02], and [03] will be red objects, where [04] through [07] will be green objects.

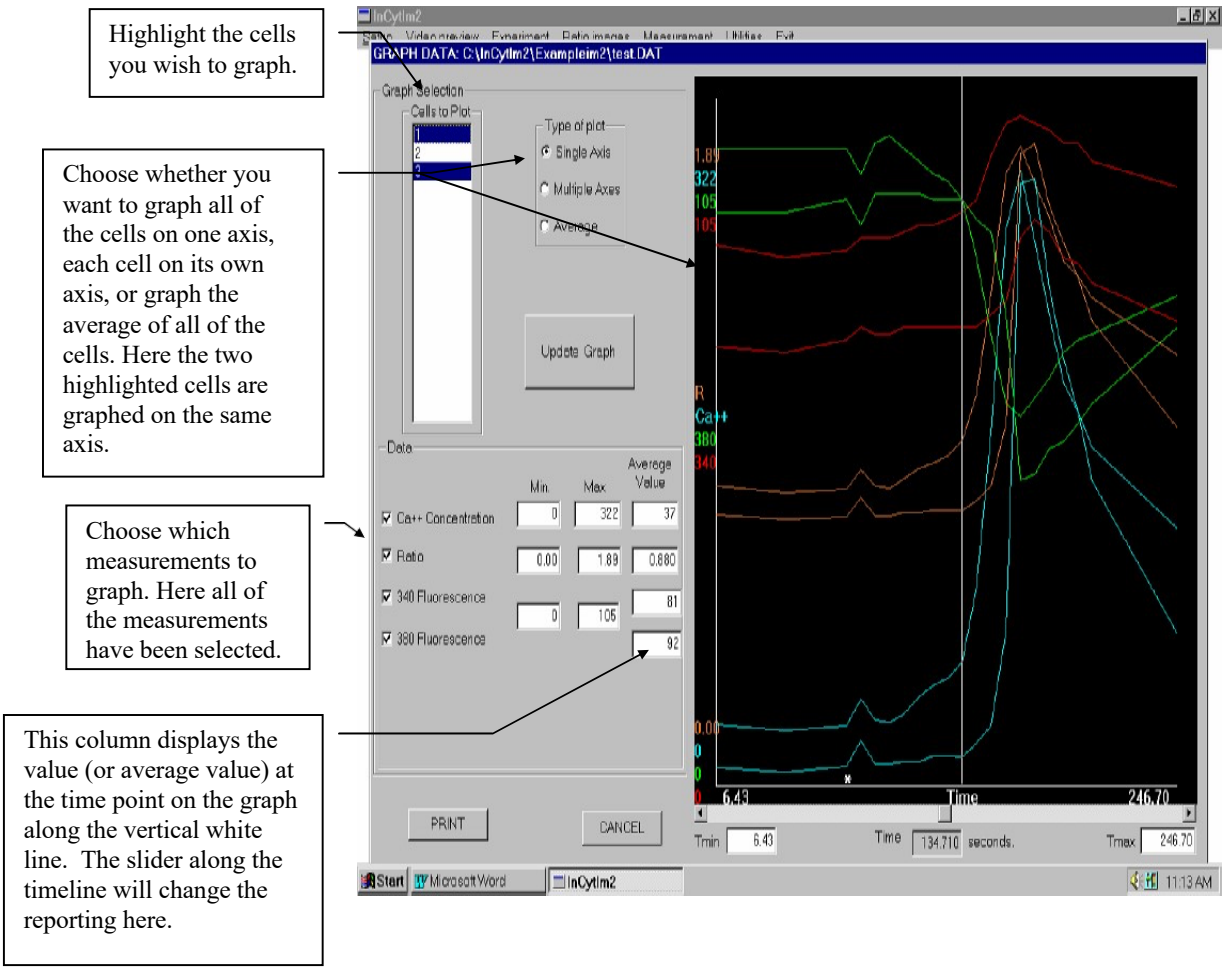
V. *InCyt Im2*™ PROGRAM: Measurement > Graph Data

3. Graph Data

a. Graphing Options

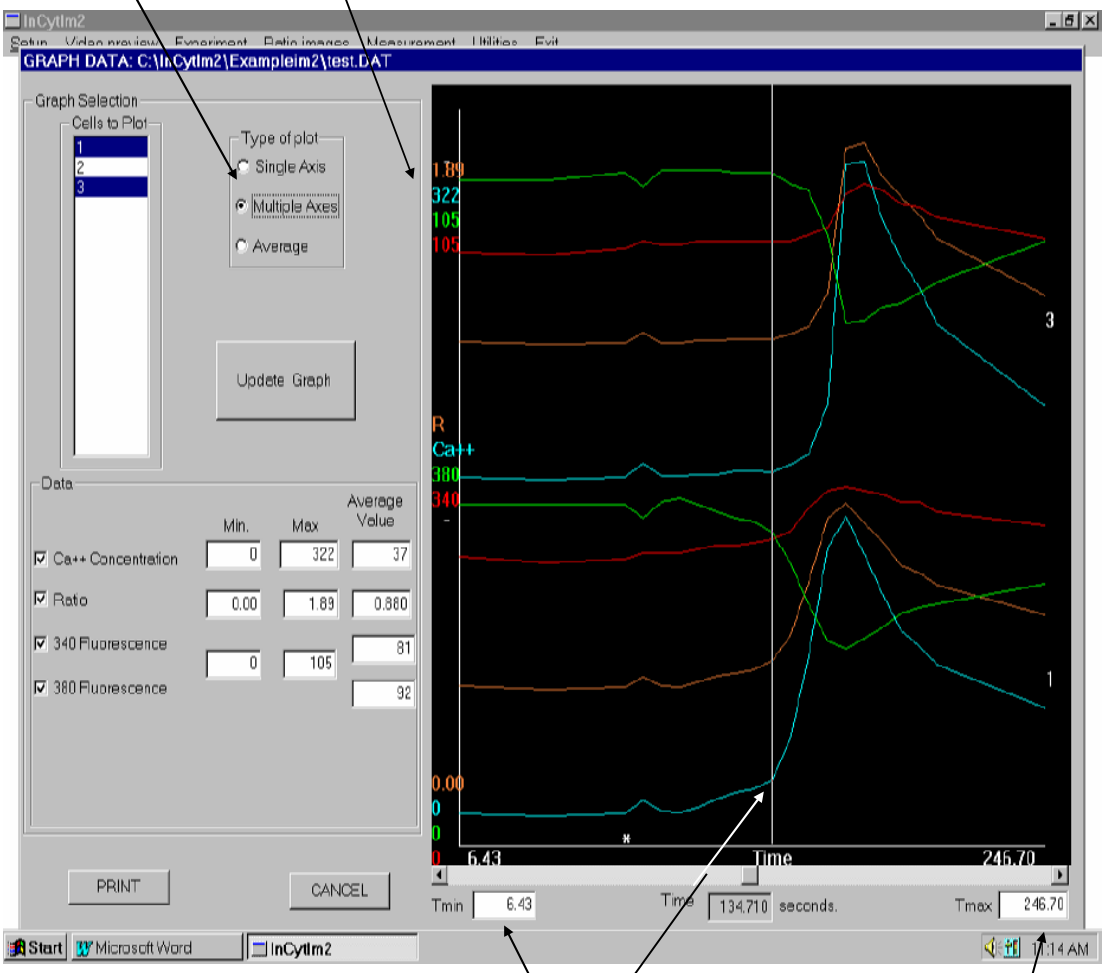


When you graph your data, you have several different options. You can graph any or all of the cells that you collected data from during the experiment. You can graph them all on the same axis, each cell on its own axis, or you can average the measurements of all of the selected cells and graph the average. The data that can be graphed is the Ca++ concentration, the ratio of 380 fluorescence to 340 fluorescence, the 340 fluorescence, and the 380 fluorescence. The average values column displays the value of each measurement (the average value if more than one cell is graphed) at the time point selected by the vertical white line on the graph. You also have the option to view the graph of the whole experiment, or to zoom in on specific time intervals.



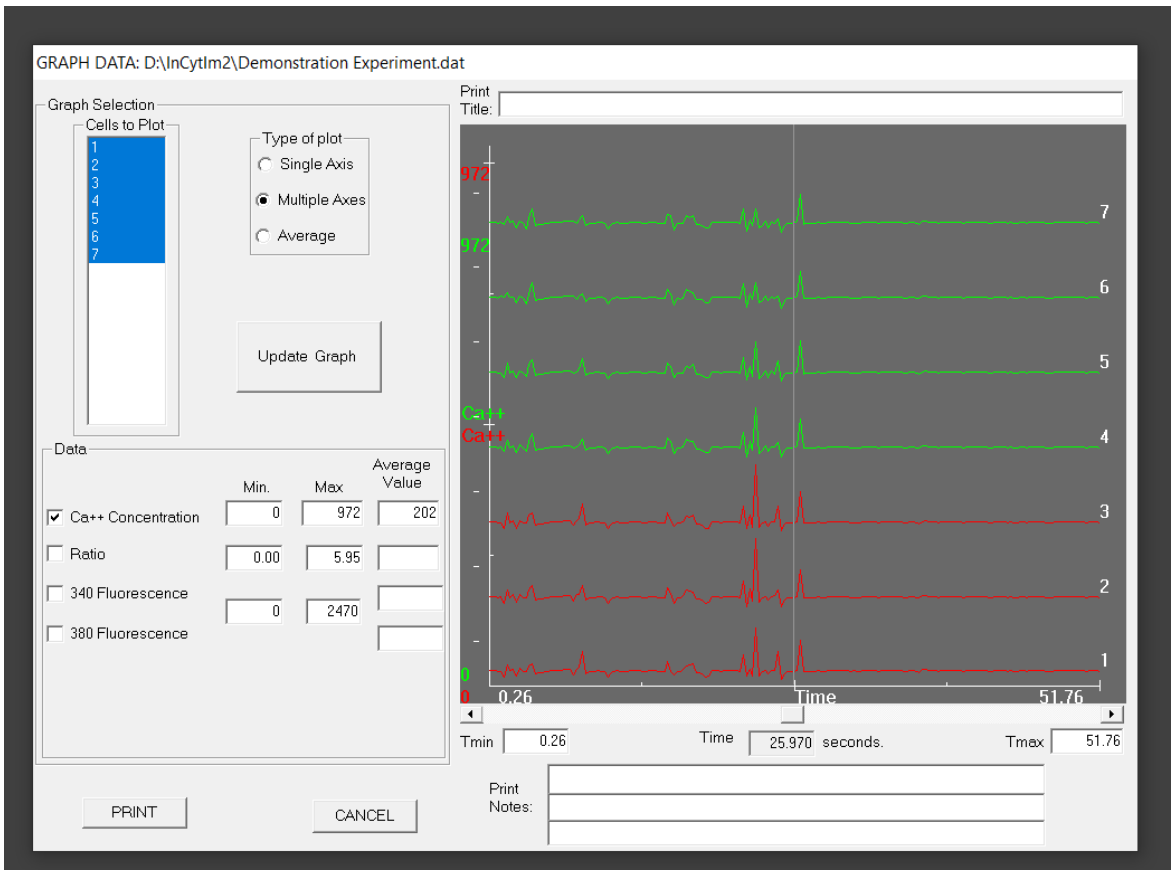
V. *InCyt Im2*™ PROGRAM: Measurement > Graph Data

b. Timing



V. *InCyt Im2*™ PROGRAM: Measurement > Graph Data

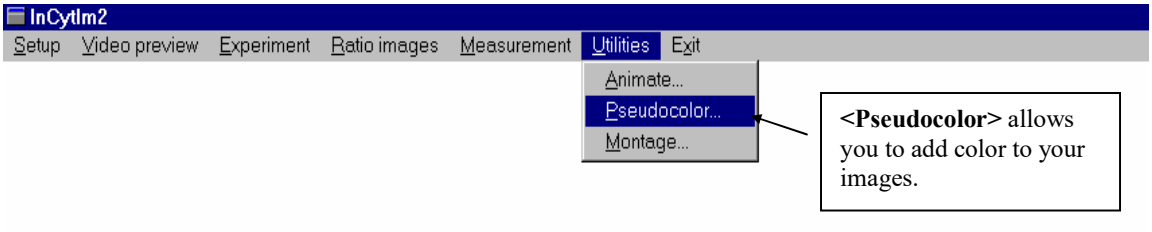
When you have selected red and green object masks, they will be graphed separately by color group:



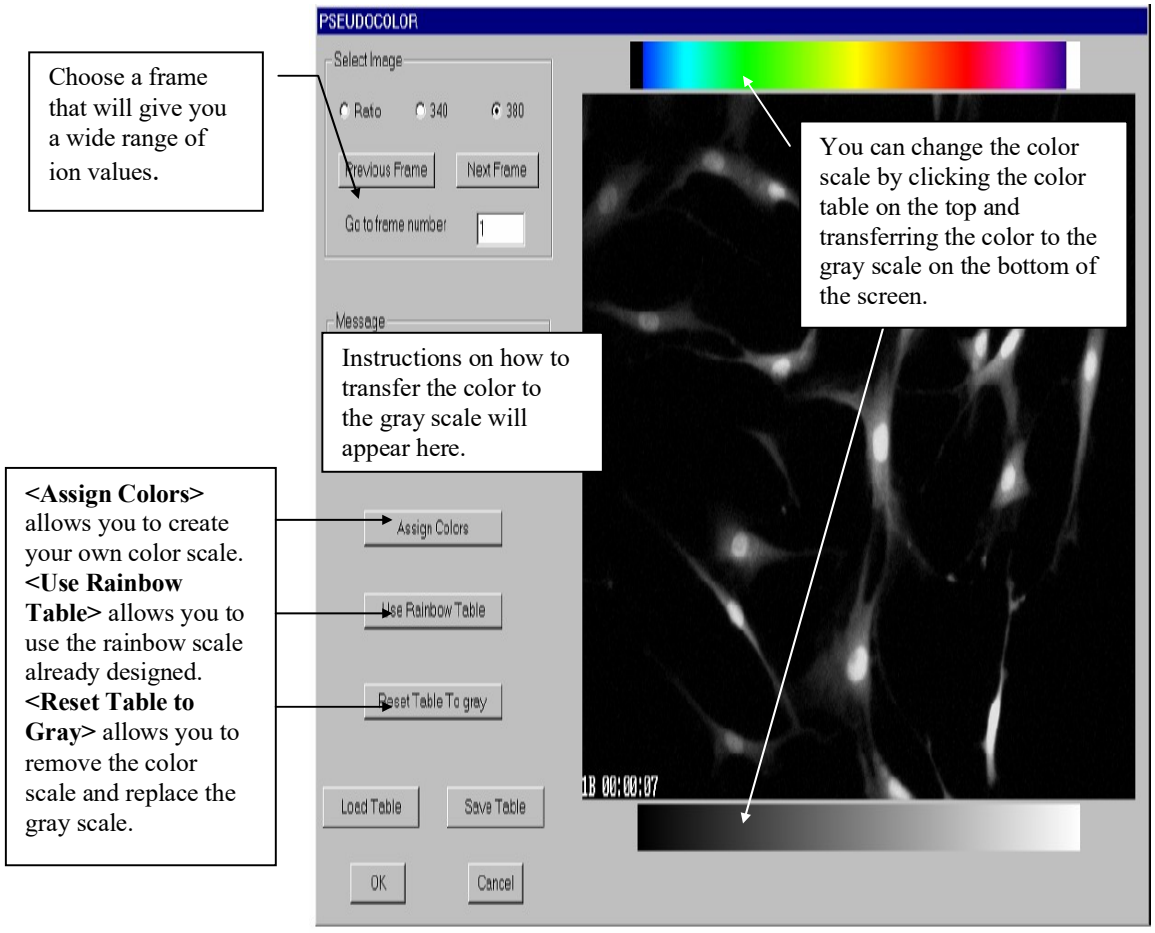
V. *InCyt Im2*™ PROGRAM: Utilities > Pseudocolor

G. UTILITIES

1. Pseudocolor

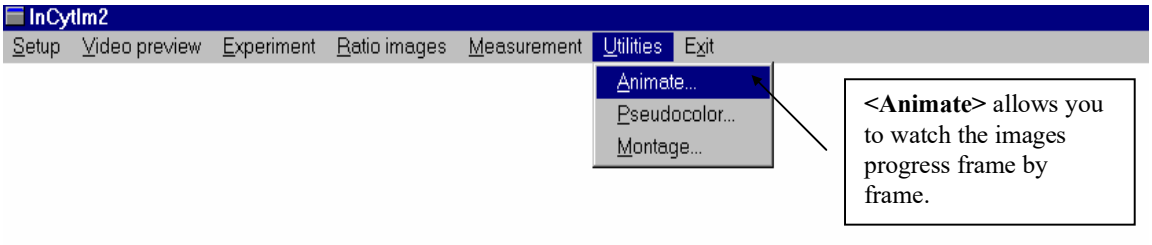


Because color changes can be easier to detect than gray scale changes, you may want to change the gray scale to a color scale. The **<Pseudocolor>** option allows you to do this. In the gray scale, the lowest levels of calcium are darkest, and the highest levels are lightest. In the typical rainbow scale, the lowest levels of calcium are blue, and the highest levels are violet, with a range of colors in between. You can also create a scale of your own by transferring the colors from the color scale to the desired position on the gray scale at the bottom of the image. Instructions on how to transfer the color will appear in the message area once you click on the rainbow scale.

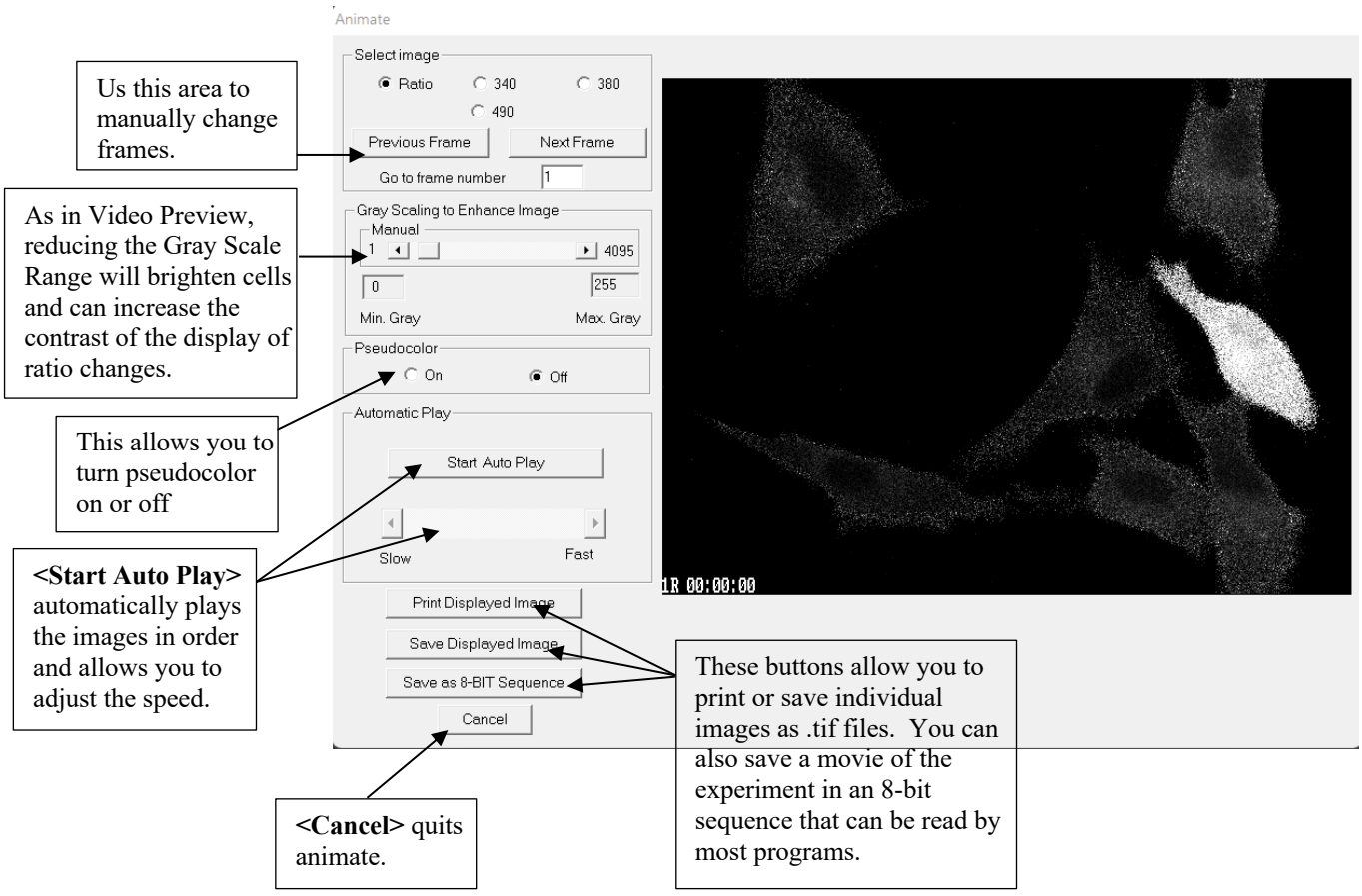


V. *InCyt Im2*™ PROGRAM: Utilities > Animate

2. Animate



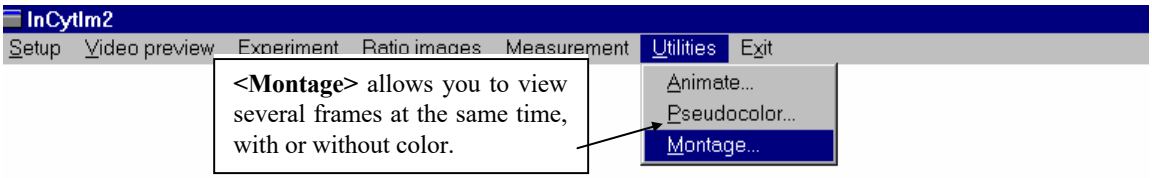
Animate is a good way to see how the changes progressed during the experiment. The animate function will replay the experiment for you frame by frame, or you can manually choose the frames you wish to view. This allows you to compare the different images that were captured during the experiment.



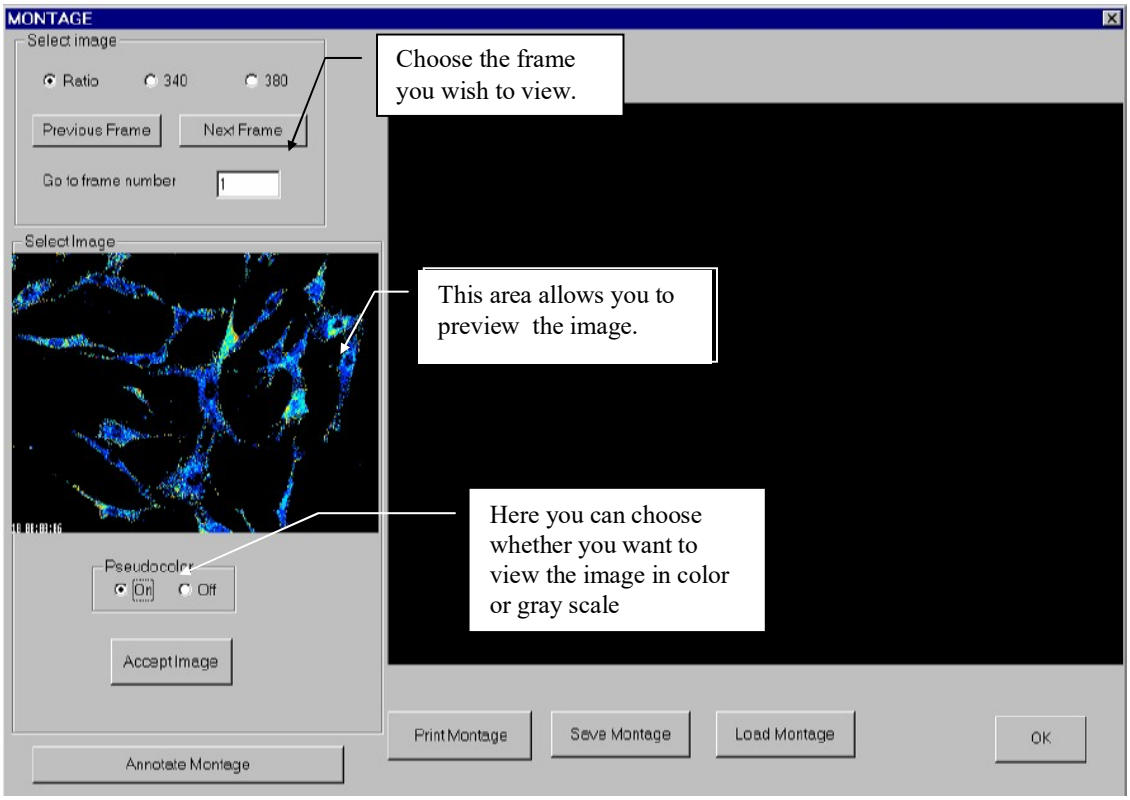
VI. Appendix

3. Montage

a. Creating a Montage

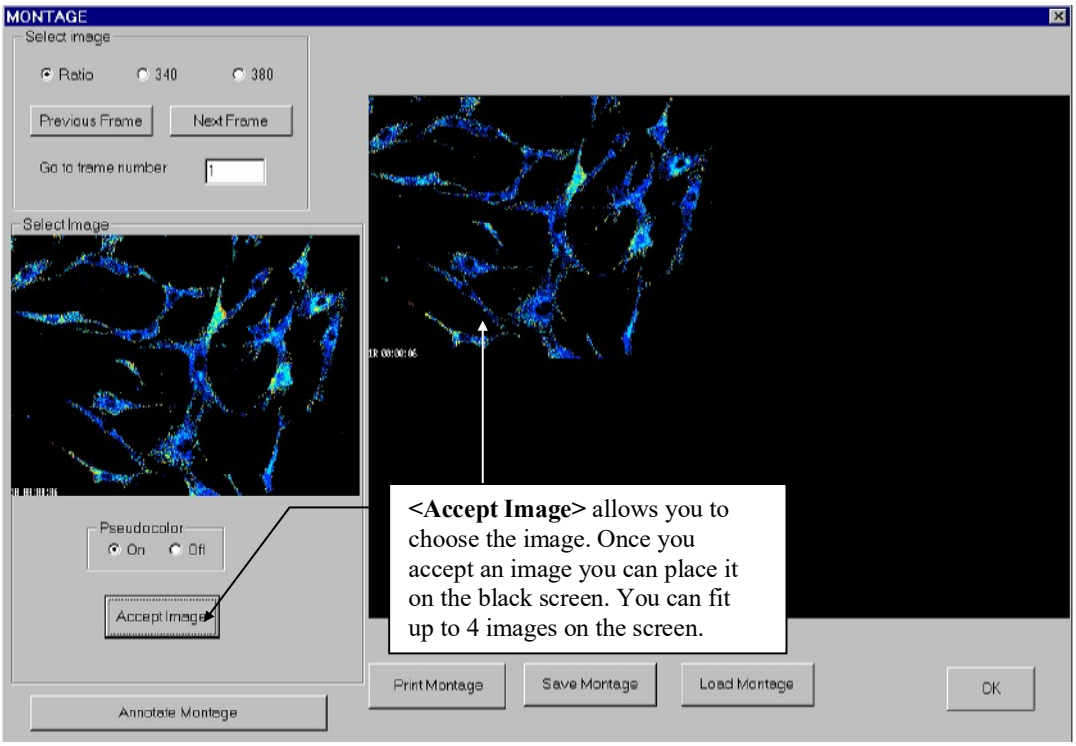


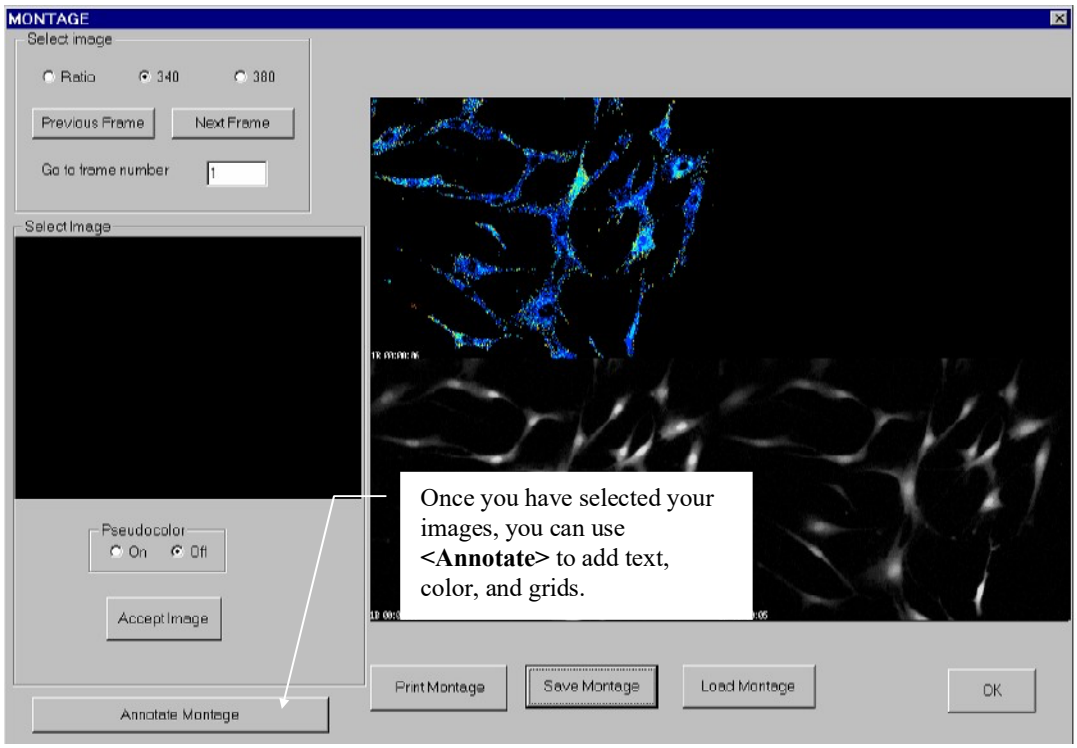
A montage is a good way to display your results. It allows you to display up to four of the images you captured from the experiment. This can help you to demonstrate the changes that occurred during the experiment or present your data in an easy to understand format. These images can be from the ratio, 340, or 380 pictures, or they can be from a variety of those 3. You can have some of the images in color and some in black and white, and you can add text to the montage. NOTE: The Observation Wavelength image is not available in Montage.



VI. Appendix

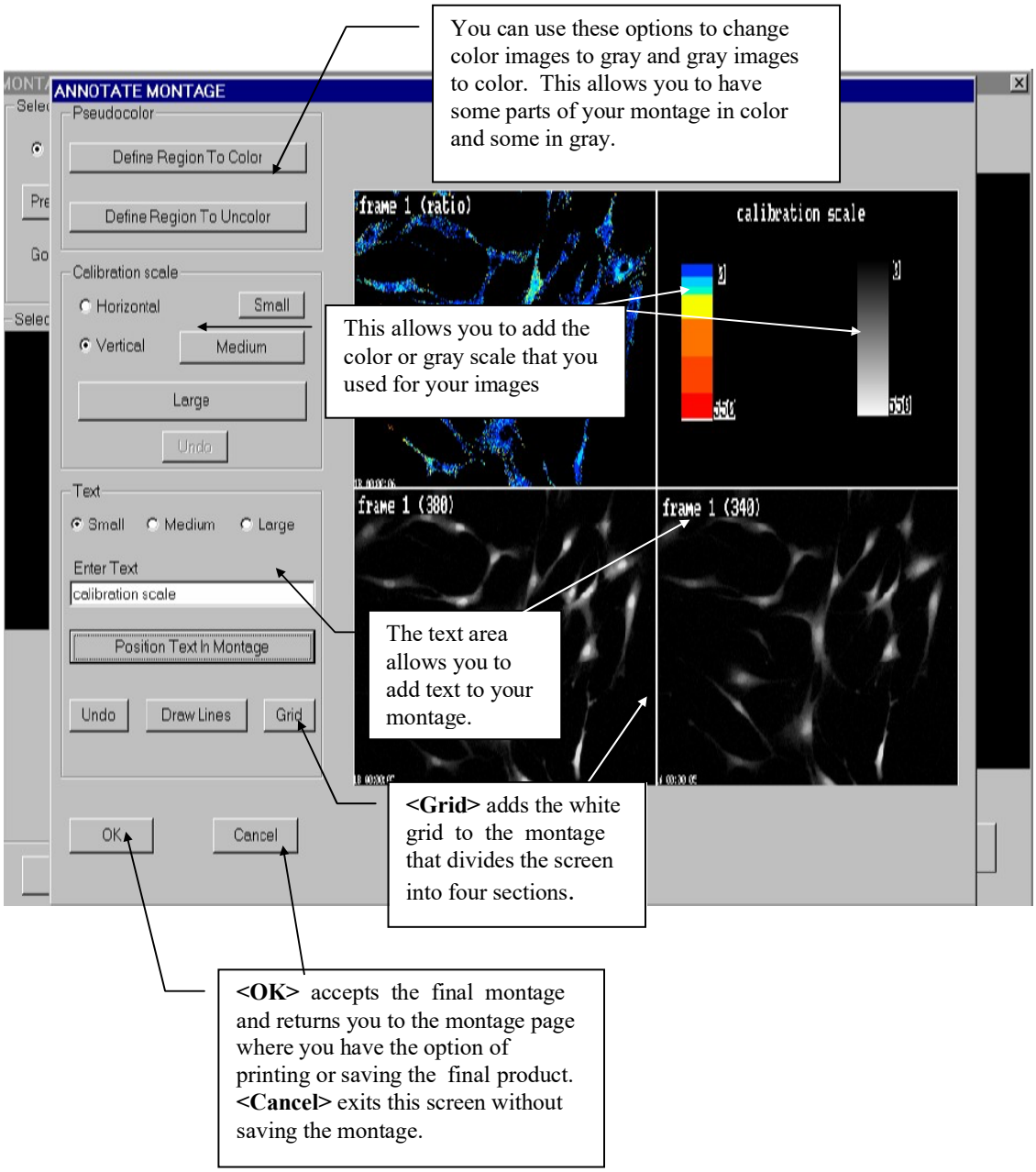
a. **Creating a Montage** (continued)





VI. Appendix

b. Annotating the Montage



VI. Appendix

A. Preparing a Calibration curve from Standard Calcium Solutions of known concentrations

1) Acquire a vial of Fura-2 penta-potassium salt (K5) from Invitrogen (catalog number F1200).
   - Dilute the 1 mg vial of K5 in 2 ml of deionized water.
   - Aliquot 50 micro liter volumes of K5 **and freeze.**

2) Acquire Standard calcium concentration solutions from Invitrogen (catalog number C3008MP). This kit contains:
   - 10 mM K_2EGTA buffered solution ("zero" free Ca^{2+})
   - 10 mM CaEGTA buffered solution (39 µM free Ca^{2+})

3) Create 1 ml of at least six standard solutions by mixing the two buffered solutions in the following proportions. **These solutions can be stored at 4ºC indefinitely. DO NOT freeze.**

$[Ca^{2+}]$ Standard nM	Zero Ca^{2+} Solution ml	39uM Ca^{2+} Solution ml
0	1	0
38	0.8	0.2
100	0.6	0.4
225	0.4	0.6
351	0.3	0.7
602	0.2	0.8

4) Remove a 50 micro liter sample of K5, thaw and put 2 micro liters of K5 in 6 small vials.

5) Add 150 micro liters of 0nM, 38nM, 100nM, 225nM, 351nM, and 602nM solutions to each of the six vials

You now have the solutions prepared that will be used to calibrate your system

6) Put a 45 micro liter sample of each calibration solution on the calibration slide in order of concentration lowest to highest.

7) From the software menu select **<Set Up>** and **<Calibration>**.

8) Select **<New Graph from Solutions>.**

9) Select **<Video preview>**.

10) Set each wavelength 340/380 to 100 millisecond exposure.

11) Slide the 100nm solution over the objective until you see a green dot in the middle of the sample; if not turn up the lamp intensity until you do; the green dot should be roughly centered in the sample.

12) Insure the binocular/photo port selector on the microscope to select photo port .

13) Click **<Check Brightness>** in Video Preview.

14) A red circular area should appear on the monitor; if the entire screen is red reduce the light intensity until you see a small circular red area; if the screen has no red pixels turn up the light intensity until you begin to see red pixels. **Note that you can change the Gray Level Range in Video Preview to affect how many red pixels you see. The camera will be saturated if you see red pixels when the Gray Level Range is set to 4,095. If this happens, definitely turn the lamp intensity down.**

15) The next step is to get the microscope objective focused on the sample; use the focusing knob to move the objective such that the red area on the monitor increases; if the entire screen turns red; turn the light intensity DOWN until a small red circular area appears; now use the focusing knob to make the red spot enlarge again; repeat the adjust light down/focus process until the red area does not enlarge when you move the focusing knob; IF YOU CONTINUE TO MOVE THE FOCUSING KNOB IN THE SAME DIRECTION THE RED DOT WILL START TO GET SMALLER. The objective is focused properly when the red circular area starts to get smaller when the focusing knob is moved in either direction.

When you look at the sample, a small green dot should be roughly centered in the sample.

16) Look at the fluorescence with both the 340nm and 380nm filters. Adjust the exposure times so that the two images have roughly the same brightness. The histogram underneath the live image can help you more easily detect whether the two fluorescence intensities are similar.

17) Place the 0nM calcium solution over the objective. Switch the wavelength to 380nm. The 380nm image for the standard curve will never get brighter than it is right now, nor will the 340nm signal get fainter. Make sure that the intensity at 340 is noticeably greater than the background intensity, otherwise the signal to noise ratio will be too low to give a good standard curve. If it is too low, increase the lamp brightness but do not change the exposure times. Make sure the 380 image does not have saturated pixels…if it does, turn the lamp intensity down.

18) As a final check on lamp intensity, place the solution with the highest calcium level (e.g., 602nM) over the objective. Switch the wavelength to 340nm and **<Check Brightness>** for saturated pixels again. The 340nm intensity is brightest at these high calcium levels while the 380 image is faint. If the 380 image is not appreciably above background, increase the lamp intensity.

19) Click **<Accept Exposure Time>** and the program will move back to the <Calibration> screen.

20) Slide the solutions slide away from the objective such that the objective sees nothing fluorescent.

21) Select **<Capture Background>**.

22) The program will automatically capture a background image and display it on the monitor; it should be what appears to be a black image.

23) Slide the 100nM calibration solution over the objective, make sure the green dot is roughly centered in the sample (do not change the focus). Enter 100 in the **Enter (Ca++)** box and click **<Capture Image Pair>**. Adjust the exposure time and capture the image pair until you get the ratio value of approximately 1.0. You need to delete the captured values before being able to capture the next. When you have achieved a ratio value of approximately 1.0, delete the final 100nM data point.

24) Slide the 0nM calibration solution back over the objective; make sure the green dot is roughly centered in the sample; do not change the focus.
- Enter 0 in the **Enter (Ca++)** box.
- Click **<Capture Image Pair>**.
- Repeat for each of the solutions.

25) When you have finished with each solution; click OK.

26) You will return to the calibration screen and be queried about saving the graph and making it the default.

Key Points:
1. The red bright spot during fixing focus must be close to the middle of the field (on the monitor). If it is not, adjust the light path of the microscope.
2. Adjust the exposure time for 340 and 380 such that the ration for 100nM calcium solution becomes about 1.0.
3. Keep the threshold setting 0 (untouched)
4. Do not disturb the dichroic mirror after calibration.
5. Fura-2 penta-potassium must be new.
6. The green spot on the drop must be at the middle of the drop.