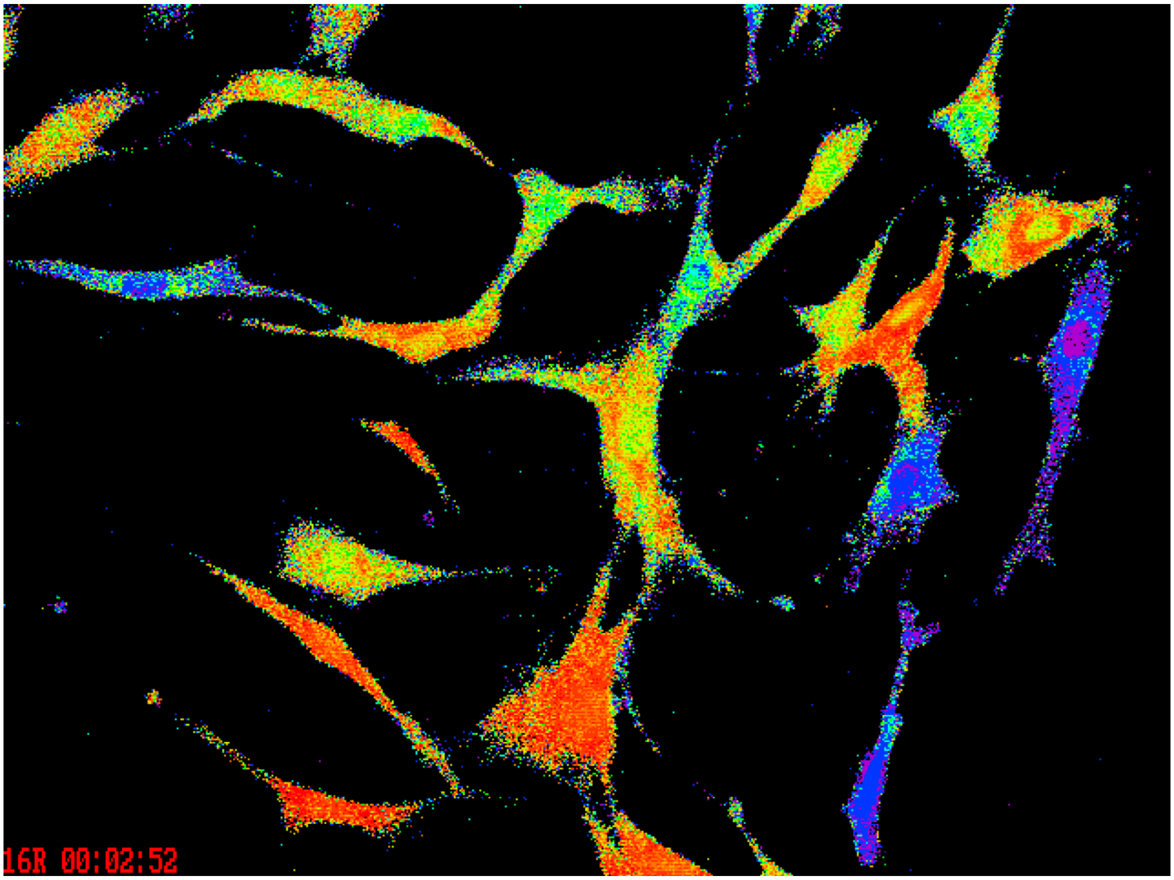
InCyt Im2™

Dual-Wavelength Fluorescence Imaging System



USER'S MANUAL
Version 4.50 for Windows NT

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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, or the accompanying Applications and Troubleshooting Guides, 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 NT®. To familiarize yourself on the use of programs in the Windows NT® 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 **<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 [ENTER REFERENCE HERE]. 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 **(minimum specifications):** Intel Pentium III 500 MHz based PC, Data Translations 3155 image acquisition and analysis board (frame grabber board), 128 Mbytes RAM, 13 Gbyte HD, 1.44 Mbyte 3.5" floppy drive, 17X CD-ROM, internal Zip Drive, network card, color graphic accelerator card, Windows NT operating system

☐ Pre-Loaded Software (disks):

- ☐ *InCyt Im2*™ Image Acquisition and Analysis Software (dual-wavelength)
- ☐ *InCyt Im1*™ Image Acquisition and Analysis Software (single-wavelength)
- ☐ Data Translation Drivers
- ☐ Windows NT® Software and Repair Disk
- ☐ Microsoft Office Small Business Edition
- ☐ Workstation manuals/software

☐ Software Security Dongle
☐ Mouse Pad
☐ Allen Wrench Set
☐ UV Goggles
☐ Standard Dish (for holding up to 6 calibration standards)
☐ Control Cable

☐ 15" or 17" SVGA non-interlaced monitor

☐ Nikon Model TS-100 microscope with trinocular head with the following accessories: **(NOTE: If you did not buy your system with our retrofitted TS-100 Microscope, a light guide adapter for your fluorescent microscope is enclosed)**

- ☐ two(2) 10x eyepieces
- ☐ ELWD condenser
- ☐ 10x phase contrast objective
- ☐ Fluor objective (base system includes 20x Plan Fluor NA = 0.5; WD = 2.1mm)
- ☐ phase slider
- ☐ mechanical stage for TS-100 microscope
- ☐ 65mm petri dish holder
- ☐ 35mm petri dish holder
- ☐ Groony™ Fluorescence Optics Module for Nikon TS-100 microscope (installed)

☐ Liquid light guide
☐ Surge Suppresser/Power Strip
☐ Diagnostic Instruments 0.55x TV Relay Lens, with parfocalizing adjustment
☐ Cohu 4920 Cooled 8-bit Low Light Level CCD Camera with power supply
☐ 300-watt xenon arc illuminator
☐ Sutter Lambda 10-C (standard) or Sutter Lambda 10-2 (upgrade) with 340 & 380 fluorescence filters
☐ User's Manual

IF YOU ARE MISSING ANY ITEMS, PLEASE CONTACT YOUR INTRACELLUAR IMAGING 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 the two foam microscope boxes and computer boxes.

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 six-foot (slightly under two meters) reach of the 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 and CDs 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 the "Application Notes" 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 Software Security Dongle to the parallel port 2 (LPT2) on the computer. Secure with the connector screws. If you have a parallel printer, connect it to the Software Security Dongle. **Do not** connect an external Zip drive to the dongle.

The system Control Cable is a "fan out" cable that has a 15-pin connector on one end and three connectors on the other end. Attach the 15-pin connector to the Data Translation Frame Grabber Board in the Workstation. This board has a male 15-pin connector and a BNC-type connector. Secure with the connector screws.

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 and secure with the screws. This cable has 15 pins in three rows.

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 300-watt Xenon Illuminator MUST be plugged into the wall or a separate surge suppresser. **The 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.
For European customers: Your Surge Suppresser will have IEC-320 output plugs and a "Schuko" plug for power. You should plug the Workstation, Monitor, and Control Box into this Surge Suppresser. We recommend that you plug this Surge Suppresser into another surge suppresser that has Schuko outputs and has an on/off switch. You will plug the Camera and the Step-Down Transformer into the Schuko surge suppresser. The 300-watt Xenon Illuminator, which also has a Schuko plug MUST be plugged into the wall or a separate surge suppresser. See the warning above. The on/off switch on the Schuko surge suppresser will allow you to turn on all other components at the same time after the Illuminator has fired.

II. SYSTEM PARTS CHECK LIST AND SET-UP

B. Setup

Step 5 -- Setting Up the Microscope (Nikon TS-100 Instructions)

Unpack all components and set them on the table. Place the Microscope stand upright on its base. Remove the eyepiece covers and install the eyepieces in the stand. The condenser lens fits underneath the microscope's light source at the top of the arm. With the large diameter side up and the Nikon label facing forward, secure the condenser lens with the silver thumbscrew on the right-hand side.

The phase slider has the phase ring(s) already installed. Place the slider through the slot in the condenser lens, making sure that the writing on the phase rings is up and the notches on the phase slider face back.

Remove the piece of foam between the objective turrett and the base. Screw the 10x phase and fluor objectives into the open holes. Repeat for any other objectives purchased for the system.

Fit the mechanical stage to either side of the microscope. The mechanical stage attaches to the fixed stage with two thumbscrews that attach to the bottom of the fixed stage.

The 35mm petri dish holder fits in the mechanical stage. If it feels loose, the two brackets on either side of the petri dish can be adjusted for a tighter fit. There is also a 65mm petri dish holder supplied with the system.

The Inverted Groony™ Fluorescence Optics Module is already installed in the microscope, and the dichroic cube is already mounted inside the microscope. The dichroic cube can be accessed by unscrewing the silver thumbscrew on top of the cube holder. For a cube to be in the proper position, the rod holding the cube should be pushed all the way in.

Step 6 -- Setting Up the Illumination System

The illumination system consists of the 300-watt Xenon Illuminator, the system Control Box, the Sutter Filter Wheel, and the Liquid Light Guide. Unpack these items.

Decide where you want to place the Microscope and the Monitor. You should place the illumination system to the left of or behind the microscope.

Set-up the Xenon 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.

Place the Control Box on top of the Xenon Illuminator. The green on/off light should face forward. Two strips of Velcro keep the Control Box secure. Plug the cord for the Control Box into the Surge Suppresser supplied with the system.

An adapter with a Dichroic Mirror Mount fits between the Illuminator and the Sutter Filter Wheel. Mount this adapter to the Illuminator by sliding the opening on the long end of the adapter over the output port of the Illuminator. Secure with the thumbscrew. The dichroic mirror holder should be facing up and the Sutter side of the adapter should be facing toward the left. 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 and the attached Lamp coupler. Adjust the height of the Filter Wheel on the stand so that the coupler is at the same height as the port on the Dichroic Mirror Mount. Slide the coupler over the Dichroic Mirror Mount port, and secure it with the thumbscrew. Tighten the wing nuts on the Filter Wheel to lock its position on the stand.

II. SYSTEM PARTS CHECK LIST AND SET-UP

B. Setup

Step 6 -- Setting Up the Illumination System (Continued)

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.

Insert the other end of the Liquid Light Guide (which should also have an 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.

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 Com2 on your computer.

Your system will come with a control cable for the camera. Place the DB15 connector on this cable into the port on the Data Translations 3155 card in the computer. This cable now fans out into multiple other connections. Connect the BNC connector labeled "8" to the BNC connector on the camera. Connect the DIN8 connector to the camera's "AUX" port. Leave all other connectors on this cable unattached.

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.

We have provided a visible-absorbing filter in one of your drop-in filter holders. This filter holder drops into the slot provided in the Lamp/Wheel Coupler. We recommend that you use this filter when conducting fura2 experiments. The filter will cut transmission somewhat, but it will prevent heat-producing light from damaging your 340 and 380 filters. It will also prevent any change in signal resulting from filters heating up during the experiment.

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

Step 7 -- Connecting the Camera

Unpack the 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.

Attach BNC #8 from the Control Cable to the camera. Attach the last remaining plug (multi-pin/white) to the "AUX" input on the camera. Plug the camera in to the Schuko surge suppresser.

II. SYSTEM PARTS CHECK LIST AND SET-UP

B. Setup

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 -- Checking Groony™ /Light Path Centering

Using a fluorescence sample you can check to make sure that the light path in the microscope is centered. Centering instructions are available in the Groony™ Installation instructions included with your manual.

To check if the light path is centered, 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. If they appear off-center, you will need to center the Groony™. The four most likely solutions to off-center illumination are:

1. First check to make sure that the cube has not moved off-center. A clear indication that the cube has slipped is the appearance of an occluded area (half moon shape) on the screen using fluorescent illumination (note that you will sometimes see what appear to be occluded areas when the room lights are on).

2. Then check the positioning of the camera. Sometimes rotating the camera in the photo port can center the image better.

3. If the image is still off center, make sure that the screws in the adapter that hold the relay lens to the microscope are all tightened evenly.

4. The last thing you can check is to make sure that the Groony™ is secured into the back of the microscope straight.

Step 10 -- If you have any problems, do not hesitate to contact your Intracellular Imaging 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 insuring 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.**
- **EUROPEAN CUSTOMERS: The Nikon TS-100 microscope requires the 230v-to-115v step-down transformer. Damage to the microscope's power supply will result from operation in Europe without this transformer.**

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 300-watt xenon lamp will burn their coatings.

III. IMPORTANT SAFEGUARDS

E. 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 C:\ drive or another storage device before starting another experiment or turning the system off. Failure to do this may result in lost data. However, for versions 4.7 and higher, it is okay to save to the D:\ drive.

F. 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.

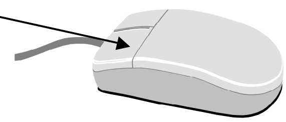
IV. KEY SYSTEM CONCEPTS AND PROCESSES

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".

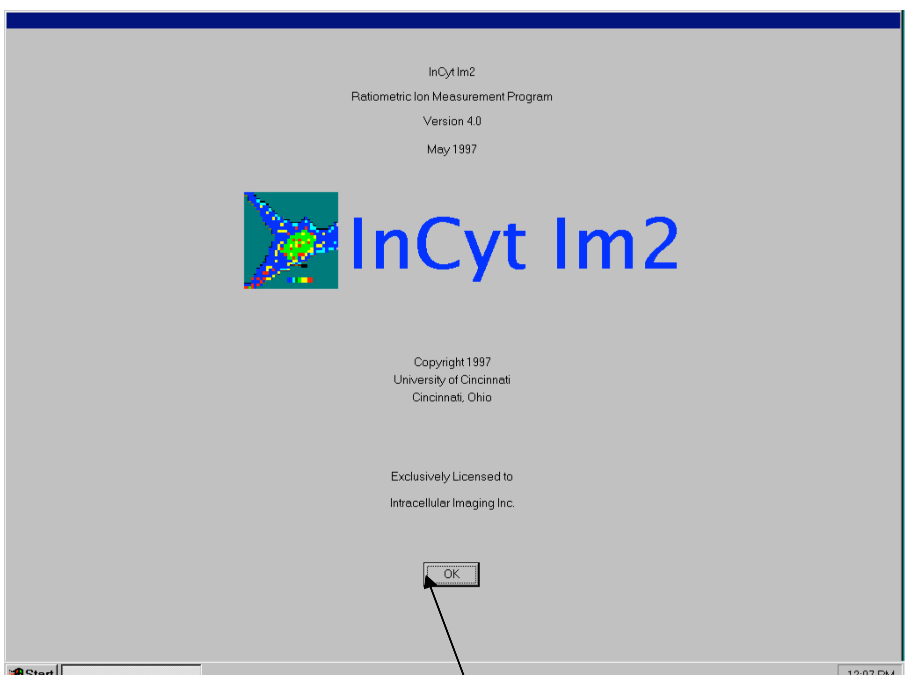
In the program description sections, brackets (< >) refer to a program menu item or button that can be accessed with a click of the LEFT mouse button.



Turning the System On

- Turn on the xenon arc lamp FIRST.
- Then turn on the other system components.
- It is recommended that all components except the 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:



Click on the <OK> button to start the program.

IV. KEY SYSTEM CONCEPTS AND PROCESSES

B. THE TWO METHODS OF MEASUREMENT

The *InCyt Im2*™ program provides two methods of performing ion-measurement experiments:

1) The first method allows you to capture and save images at both excitation wavelengths. The pairs of images at each time point are then ratioed and measured at a later time. This method, since it consumes memory or hard disk resources, limits the number of time points you may capture. However, this method allows you the maximum amount of analytical flexibility, because the images are saved for multiple analysis runs and are available for publication. This method is called **<Measure Ion After Experiment>**.

2) The second method of conducting an experiment is called **<Measure Ion During Experiment>**. In this method, the user can measure ion concentration data during the experiment, but cannot save the images. The benefits of this method are a) the data for each object are calculated and graphed "on the fly", so you can monitor the response as the experiment proceeds, and 2) you are not limited in the total number of measurements that can be taken during any one experiment, because the system is saving data only, not large image files. On the downside, this method requires that the user pre-define which cells or cell areas are to be analyzed. The response data for any cell(s) not chosen for analysis are lost. During the experiment, the system captures a pair of images (one at each excitation wavelength, ratios the image areas that have been pre-defined (these areas are called "objects"), measures the ion concentrations immediately, and then discards the images.

C. MEMORY (RAM) IMPACT ON EXPERIMENTS

Your system has been delivered with 128 megabytes of RAM (Random Access Memory). In **<Measure Ion After Experiment>** mode, 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. With 128 megabytes of memory, and no other programs running, the system can save about 106 full-size (480x640 pixels) image pairs or 256 quarter-size (240x320 pixels) image pairs during an experiment. If the user chooses to save the images directly to disk, the user can capture up to 256 image pairs of any size.

D. 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 Cohu 4920 camera used in the *InCyt Im2*™ system is a cooled "integrating CCD" camera, meaning that it can integrate the exposure over the time set by the user. Exposure times can range from 33 milliseconds/frame (30 frames/sec) to 5 seconds/frame. Because the camera is cooled some of the inherent noise in the camera is eliminated.

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.

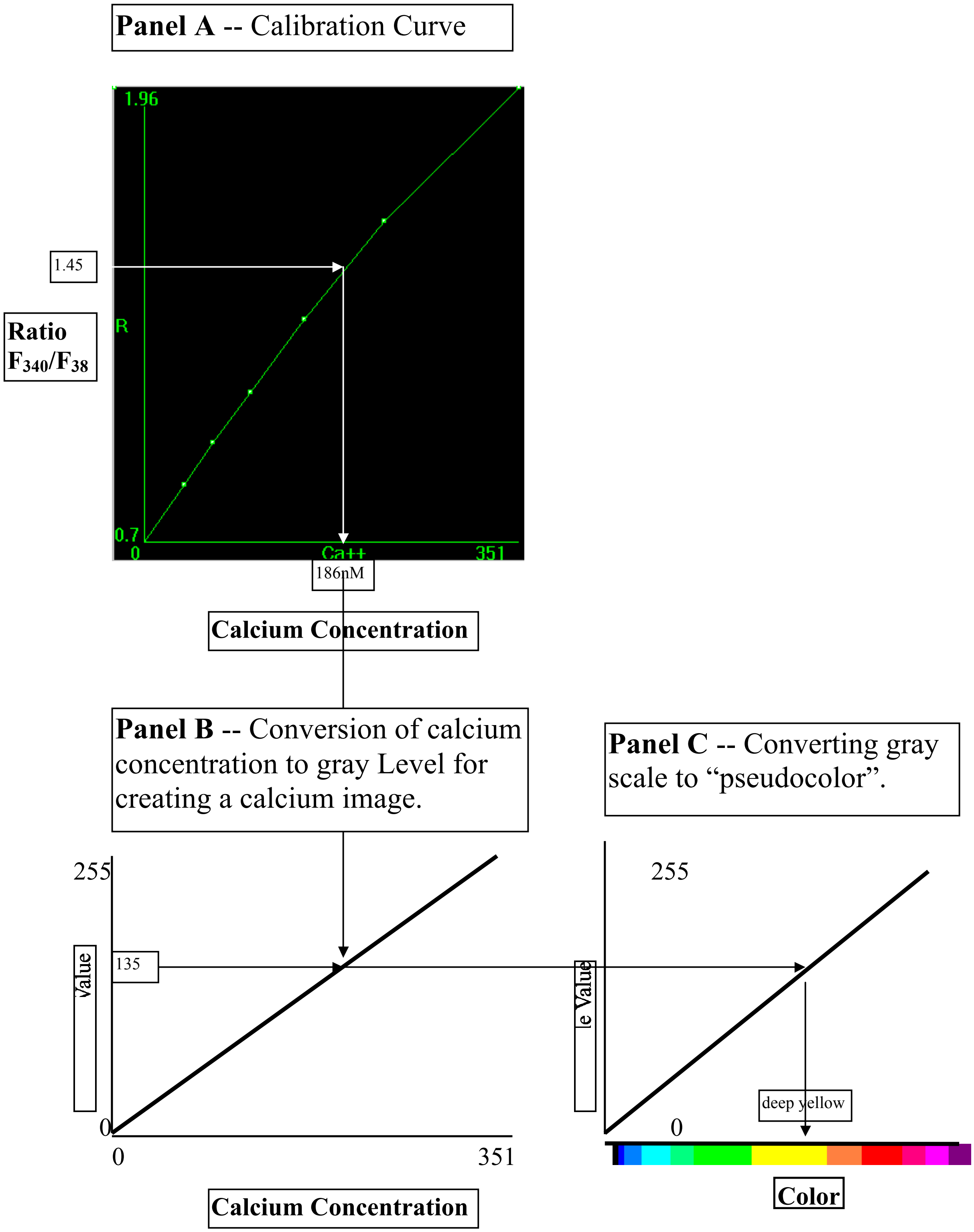
E. CONVERTING FLUORESCENCE TO ION CONCENTRATIONS AND CREATING IMAGES.

The diagram on the following page 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.

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.

E. CONVERTING FLUORESCENCE TO ION CONCENTRATIONS AND CREATING IMAGES.

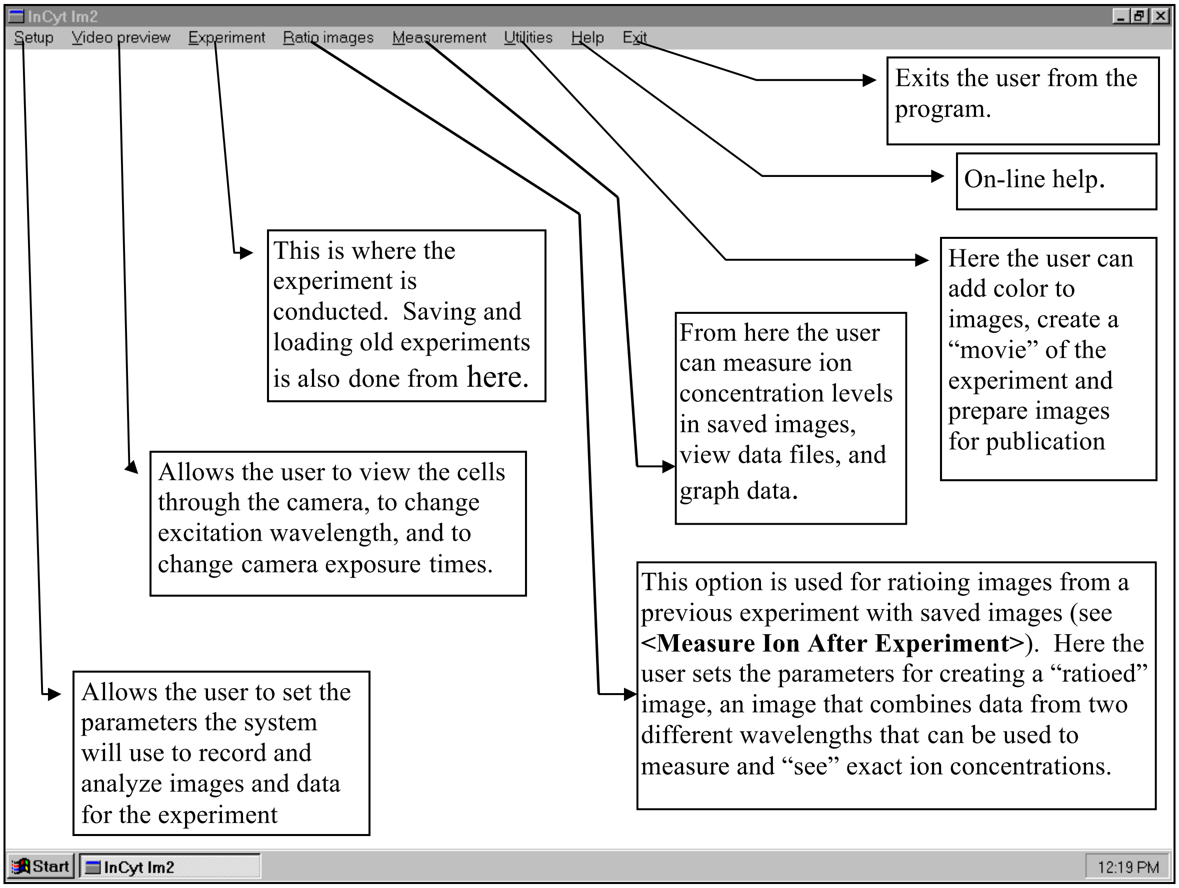


Note: All figures used in graphs are for demonstration purposes only.

V. *InCyt Im2*™ PROGRAM

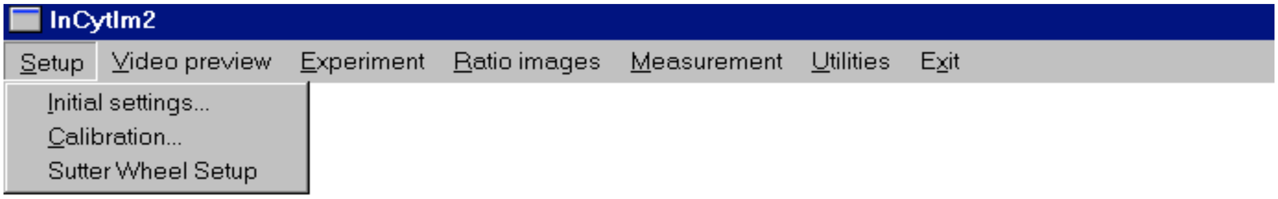
A. MAIN MENU

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



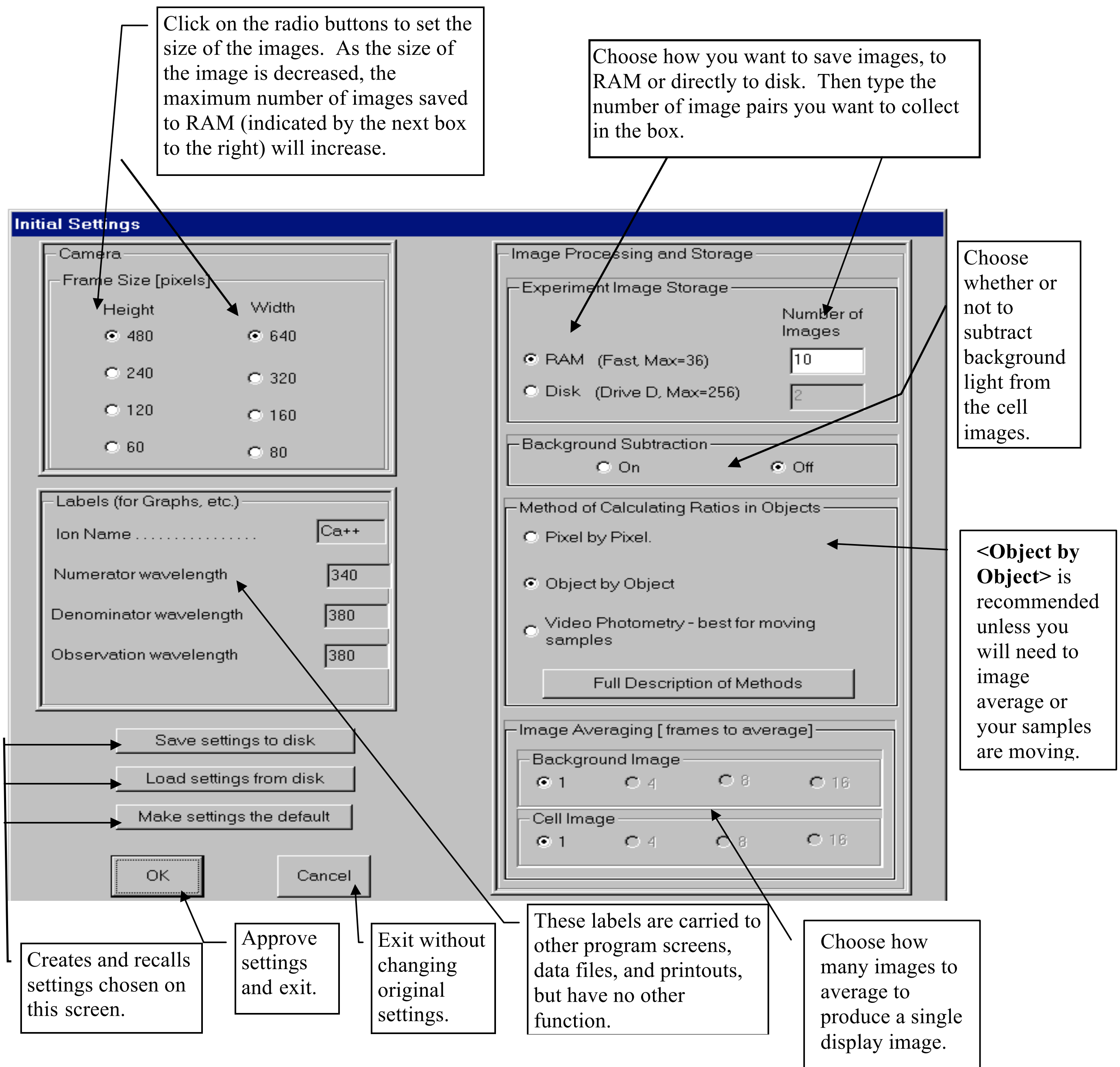
For simplicity reasons, the following discussion of the program will assume that you are running calcium studies using the dye Fura-2, and will refer to measuring "ion concentrations". The *InCyt Im2*™ program is extremely versatile and can be used to measure a range of other ions, as well as other fluorescent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

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.



V. *InCyt Im2*™ PROGRAM

B. SETUP

1. Initial Settings

a. Frame Size

The "frame size" affects the size of the image, but NOT the resolution of the image. Thus, when you select a smaller frame, you will see fewer cells. Because a larger frame size takes more memory, the frame size 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 about 307,200 bytes of data, or 307KB, 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 1MB.

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. If you will be running the experiment in **<Measure Ion During Experiment>** mode, choose the full-frame size, 480x640 pixels.

b. Number of Images

The number of images you can acquire is only limited when you are running the experiment in **<Measure Ion After Experiment>** mode. If you run the experiment in **<Measure Ion During Experiment>** mode, the system does not save the images, so your selection here will be ignored.

If you choose to **<Save Images to Disk>**, you can acquire image pairs for up to 256 time points for any size image. The images will be 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.

> 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 RAM in your workstation. Systems are standardly delivered with 128 megabytes of RAM. With 128 megabytes of RAM, you can acquire about 106 full-size image pairs (480x640 pixels), along with the associated 106 ratioed images. You can capture up to about 256 image pairs with quarter-size images (240x320 pixels). No matter how much RAM your workstation has, the maximum number of image pairs is 256.

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.

B. SETUP

1. Initial Settings

c. Labels

These fields do not have a direct functional affect 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.

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.

> **NOTE**: The filter corresponding to the **<Numerator Wavelength>**, (in this case 340nm) should be placed in the Filter Changer filter wedge on the **RIGHT**-hand side as you face the system. This is opposite of the way the wavelengths are displayed in **<Video Preview>** (see the **<Calibration>** discussion below) -- in **<Video Preview>**, the **<Numerator Wavelength>** is displayed on the left.

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.

B. SETUP

1. Initial Settings

e. Method of Calculating Ratio

The choice of method here is most important if you plan to **<Measure Ion During Experiment>**, where you choose the cells you wish to measure before beginning the experiment. If you plan to **<Measure Ion After Experiment>**, your choice will not affect anything until you measure the ion, after the experiment is over.

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 is required for image averaging.

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 may produce more accurate results when the fluorescence signal is very weak.

3) **<Video Photometry>**: In this method, each object is treated like a single 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.

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.

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.

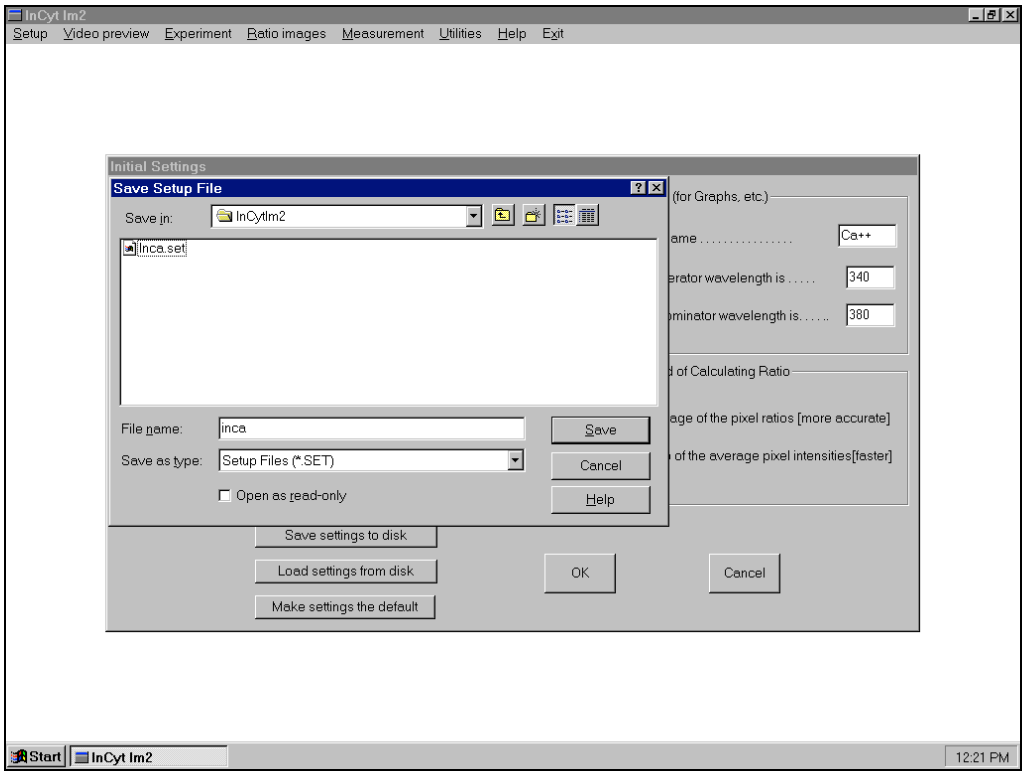
*This is equal to about 880 milliseconds per image pair -- the extra time is required to a) dampen any vibration in the filter changer before taking the image, and b) to process the images.

B. SETUP

1. Initial Settings

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"**.



h. <OK> and <Cancel>

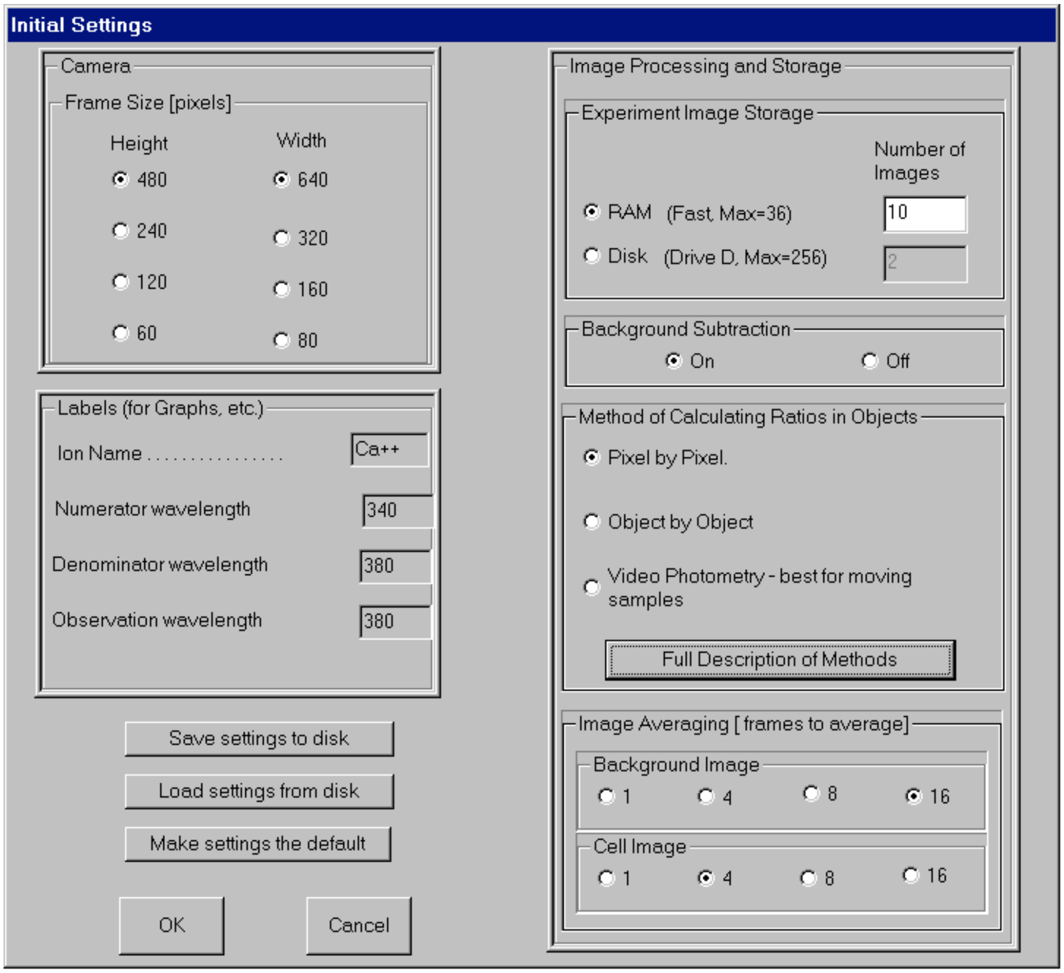
You must click on one of these buttons to exit the Initial Settings Screen. If you approve of the settings you have chosen, then click on **<OK>** and the settings will be held in memory for the experiment. **<OK>** does NOT save the settings to a file. If you exit the program, the settings will revert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

B. SETUP

1. 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.



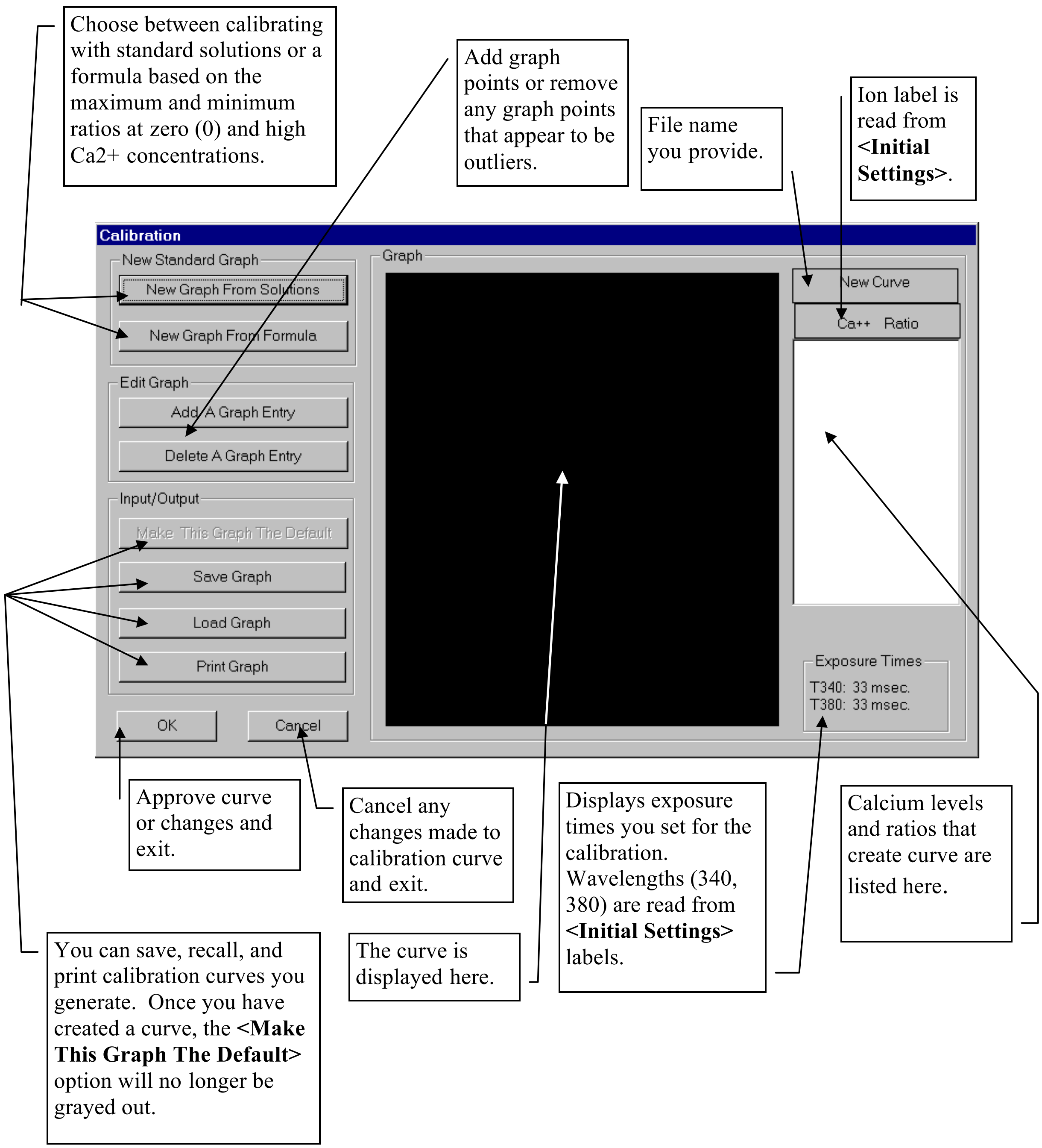
These settings indicate:

1) Full-size images (480 x 640 pixels)
2) Images will be saved to RAM. Although the system could save up to 106 image pairs, only 10 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 to reduce noise.
5) In calculating 340nm/380nm ratios (and therefore Ca^{2+} levels) for an object, each pixel will be ratioed individually and then the average of these ratios will be calculated. This option allows the user to image average.
6) To get a clearer reading on the background, sixteen(16) images will be taken of the background and averaged.
7) To reduce noise during the experiment, four (4) images will be averaged at each time point for each wavelength.

B. SETUP

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 both wavelengths.



B. SETUP

2. 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 for many ions. 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. 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 Applications Manual 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.

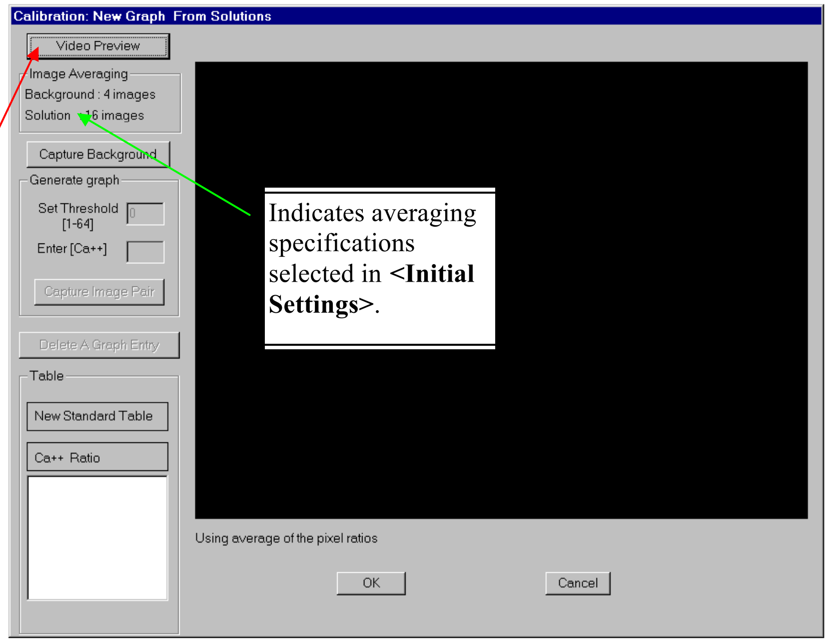
The graph-generation screen (shown next page). With this screen and those for other multi-step processes, the program prompts the user through the steps by highlighting only those steps that are available to the user at any given time. Move from the top to the bottom through the screen.

B. SETUP

2. Calibration

a. <New Graph From Solutions>

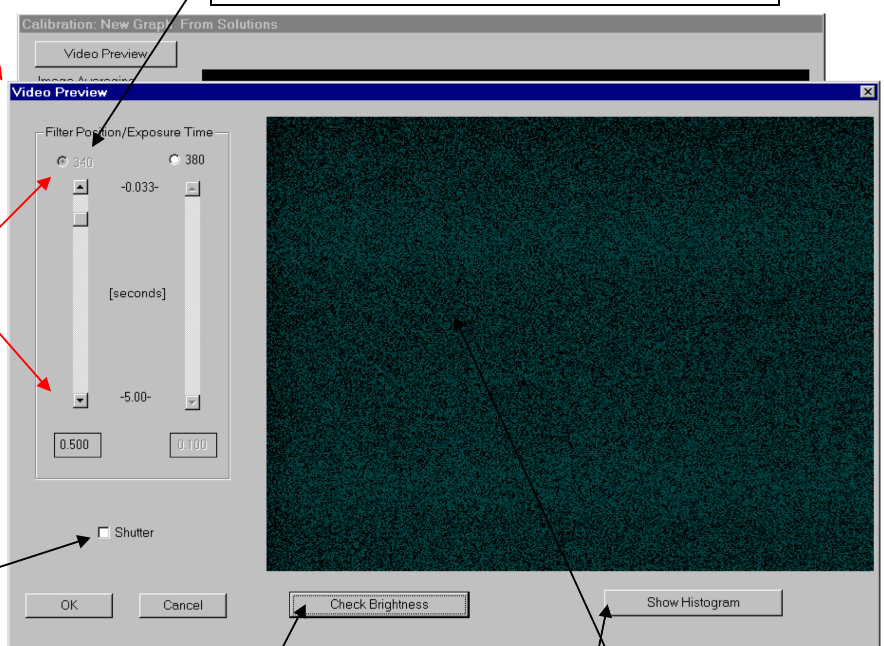
STEP 1: Go to **<Video Preview>** to set lamp intensity and exposure times.



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

Radio buttons switch filter wheel between 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. Shutter is engaged if an "X" appears in the box.

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 solutions, this will be a solid field between gray and white.

B. SETUP

2. Calibration

a. <New Graph From Solutions>

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

DARKEN THE ROOM. Place the 100nM standard solution (containing Fura Acid -- see Application Manual) on the stage and adjust the objective until it is touching the bottom of the coverslip. 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 equally bright at both wavelengths. Use the radio buttons at the top of each bar to switch between wavelengths.

The exposure times can vary between 0.033 seconds (33 milliseconds) and 5 seconds. Start off with 380nm at a 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 4:1 ratio on a new system. Over time, this ratio will start to creep up, because as the lamp degrades, it loses 340nm intensity first. (See the Troubleshooting Guide for information about when your lamp should be replaced. For our example, the integration times were set at 0.500 seconds for 340nm and 0.100 seconds for 380nm, for an integration ratio of 5:1.)

Once the integration times are set so that for 100nM Ca^{2+} the light intensity on the monitor does not vary appreciably as you switch between wavelengths, place the 0nM calcium solution over the objective. Switch the wavelength to 380nm. The 380nm image will never get brighter than it is right now. Hit the **<Check Brightness>** button to make sure that no pixels are saturated. Saturated pixels appear RED. If you see any red pixels, turn the lamp down until they disappear.

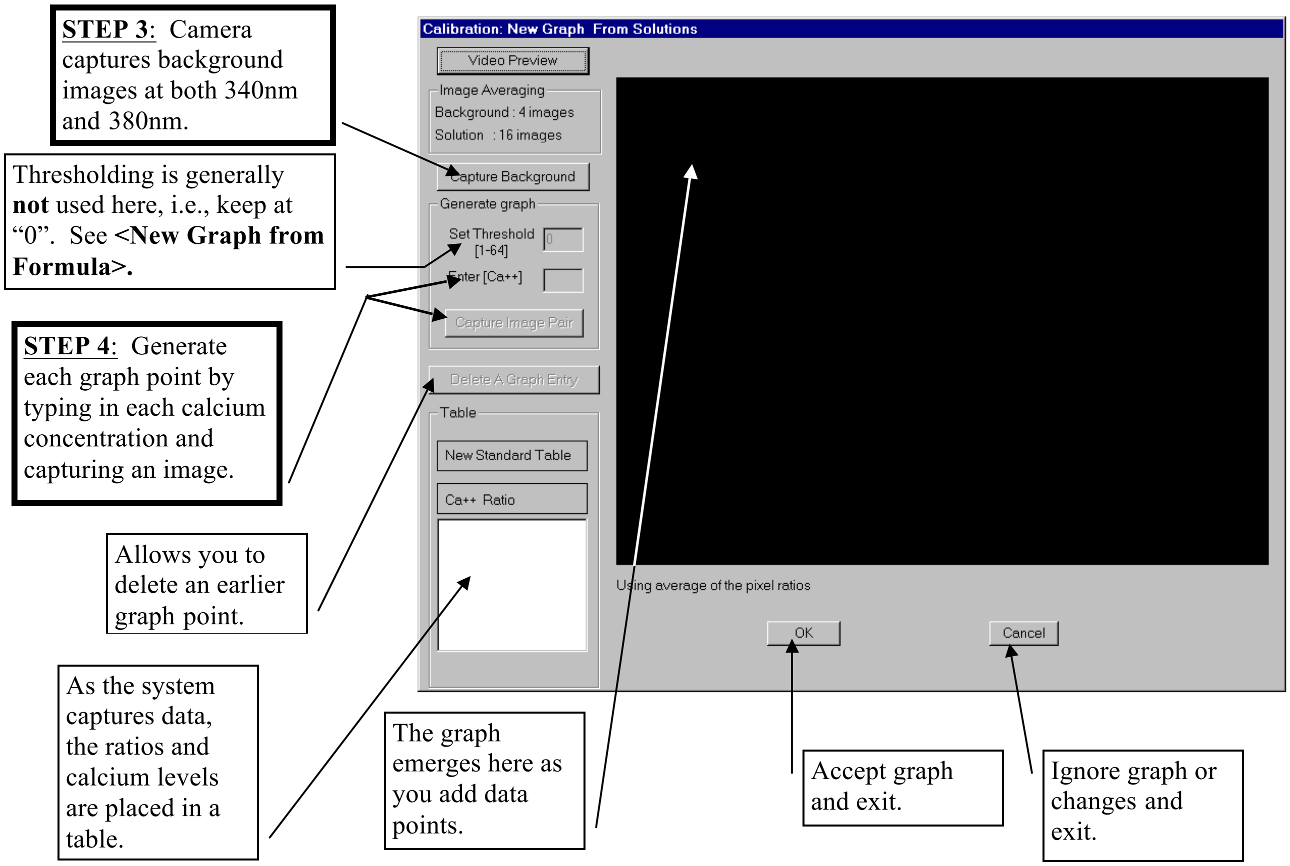
As a final check on lamp intensity and exposure times, place the solution with the highest calcium level (e.g., 351nM or 602nM) over the objective. Switch the wavelength to 340nm and **<Check Brightness>** for saturated pixels again. The 340nm intensity is brightest at these high calcium levels.

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

B. SETUP

2. Calibration

a. <New Graph From Solutions>



<u>Step 3 -- Capturing a Background</u>

Before clicking this button, defocus the microscope or remove the standards dish from the scope so that you are capturing a non-fluorescent field. Capturing the background may take a moment if you have decided, as we did in this example, to average many background images. Both a 340nm and 380nm background are captured, as the exposure times for the two wavelengths may be different.

<u>Step 4 -- Generating the Graph</u>

Refocus the microscope on the 0nM solution. Type "0" in the **<Enter [Ca++]>** box and hit the **<Capture Image Pair>** button. The system takes both images, averages images if specified, and calculates the ratio. The calcium concentration and calculated ratio are written in 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 (a ratio close to 1:00:1) between the two wavelengths at 100nM, in general the ratio will be below 1.00 for calcium values below 100nM and above 1.00 for calcium above 100nM.

B. SETUP

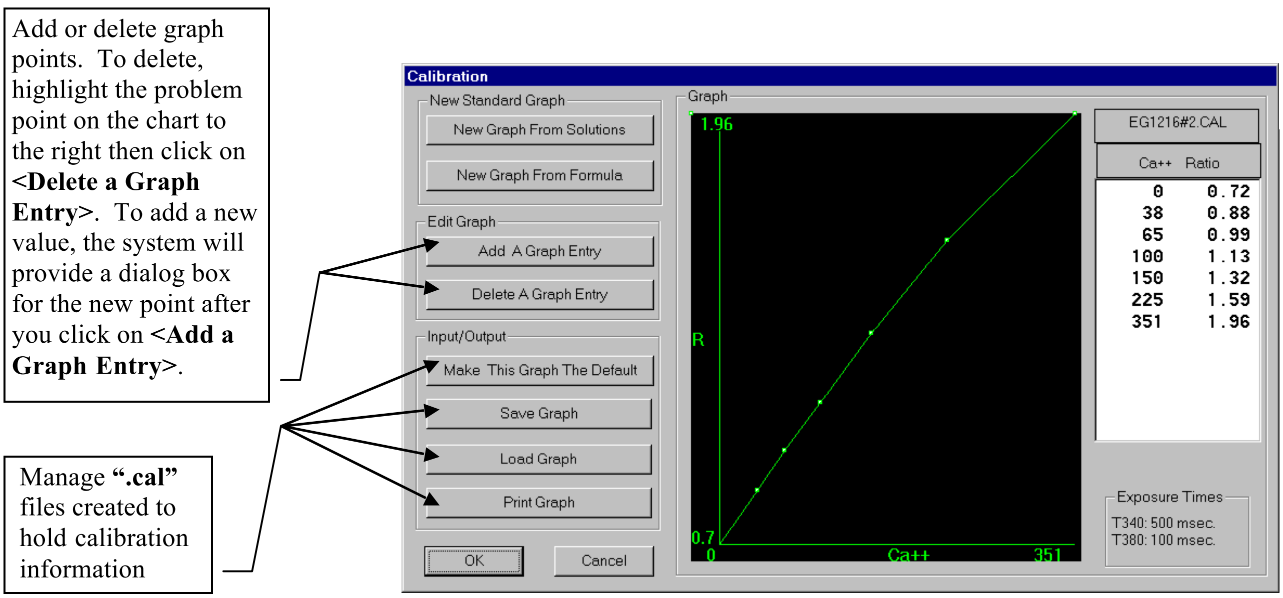
2. Calibration

a. <New Graph From Solutions>

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.



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.

B. SETUP

2. Calibration

a. <New Graph From Solutions>

<u>Step 4 -- Generating the Graph (continued)</u>

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 3.00 to 4.00 from 0nM to 351nM, and a greater range if using 602nM or more concentrated solutions.

> **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.

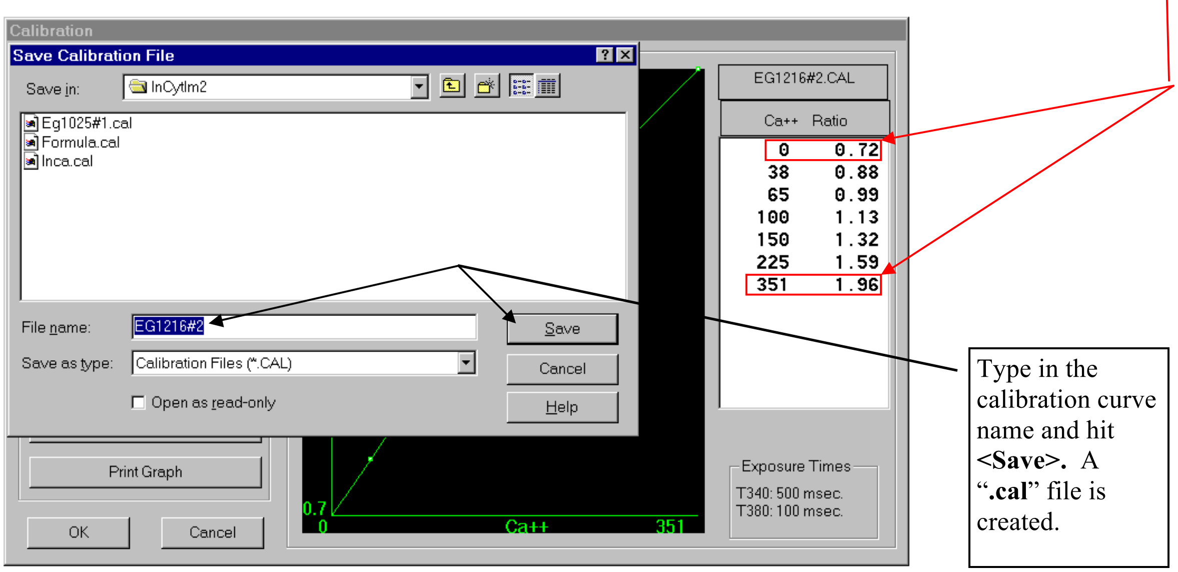
<u>Step 5 -- Print and/or Save the Graph</u>

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 data in the file **“inca.cal”**. 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:



<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 <u>without saving</u> any of your work. Be careful.

B. SETUP

2. Calibration

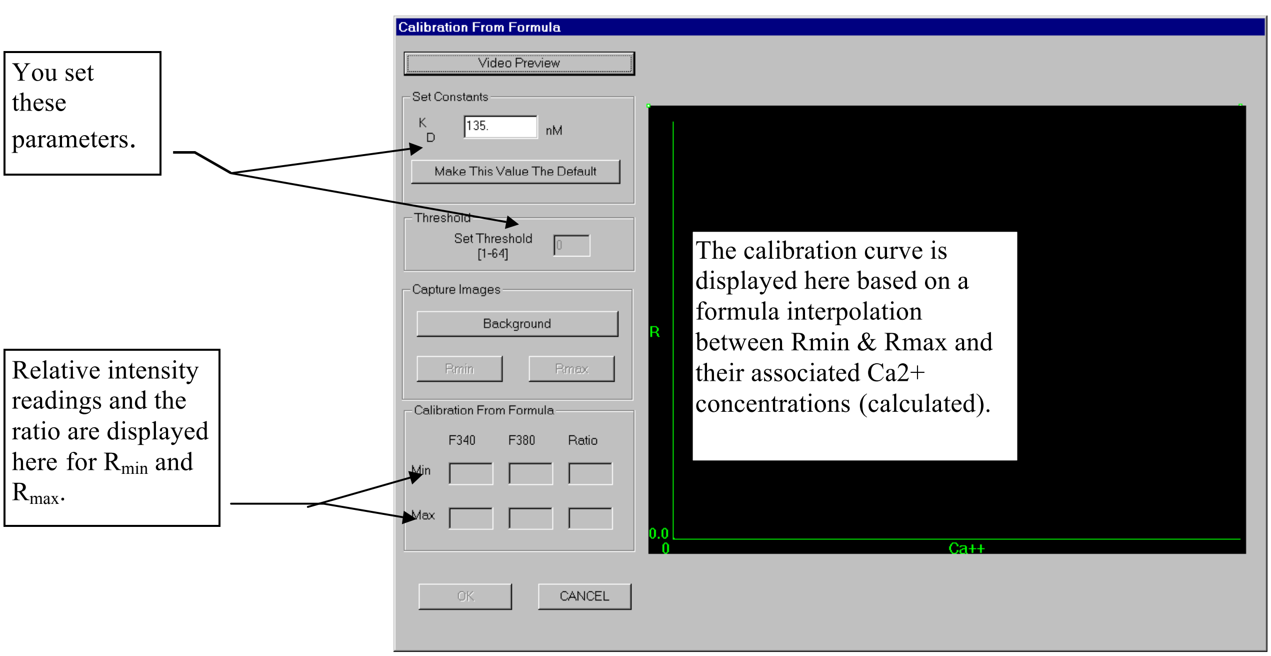
b. Creating a <New Graph From Formula>

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, <u>260</u> 3440 (1985). R_{min} and R_{max} can be measured either from two standard solutions (e.g., 0nM & 1<u>m</u>M, 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 using the **<Measure Ion After Experiment>** method, then generate a new curve from the experimental sample.

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



B. SETUP

2. Calibration

b. Creating a <New Graph From Formula>

<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 insitu measurement, add calcium-free extracellular buffer containing a calcium ionophore (e.g., ionomycine or BrA23187) plus sufficient EGTA to bring the [Ca^{2+}] to close to zero. Now click on **<R_{min}>**.

<R_{max}> If building the formula with standard solutions, focus on a 1mM or 2mM calcium solution. If conducting an insitu 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 1-2mM.

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.

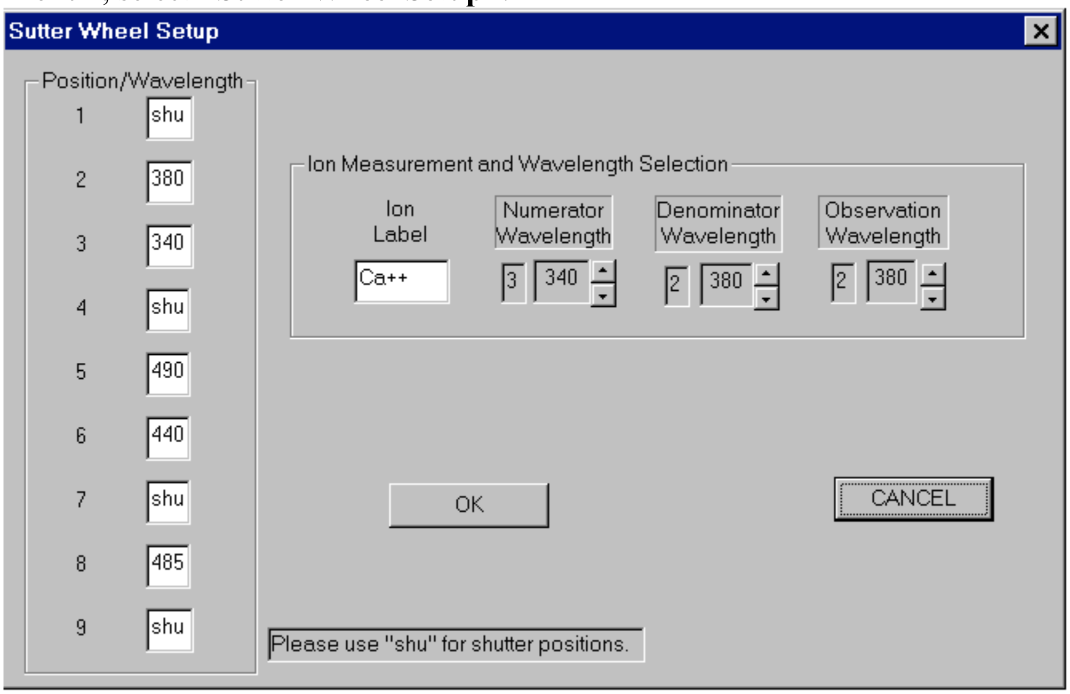
<Cancel> Exits this screen <u>without saving</u> any of your work. Be careful.

B. 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.

PLEASE NOTE: The system will not allow you to place the same position in two locations. If you only have two filters in the wheel now, a dummy numerical entry has been placed in Position 8, which is designated as the observation wavelength (although a shutter is actually in this position). If you only have two or three filter in place, and you need to switch around the numerator, denominator, or observation wavelengths, you must temporarily put another dummy numerical entry in another position. This will give you the flexibility of switching positions by using the up/down arrows.

B. SETUP

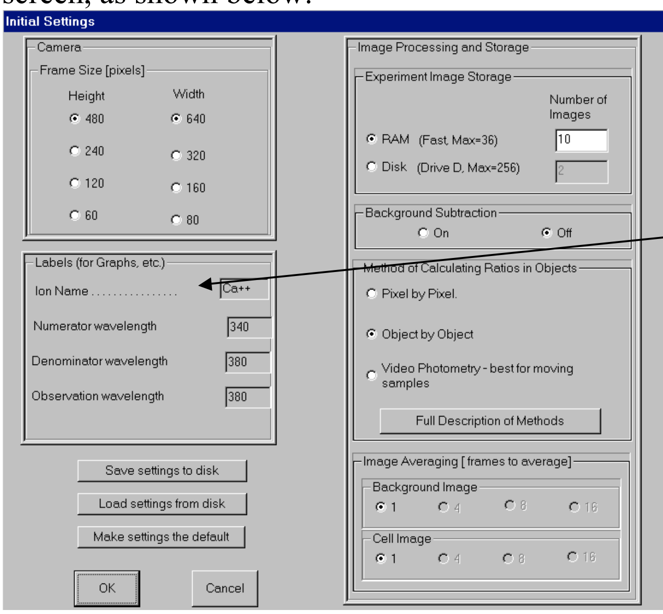
3. Sutter Wheel Setup

a. Program notes

> **PLEASE NOTE**: When using the Sutter 10-C in switching speed 2, we recommend that you **DO NOT** use filter position #5 for a Measurement Wavelength. A bug in the Sutter 10-C hardware does not allow complete control of movement to filter position #5 in speed 2.

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:



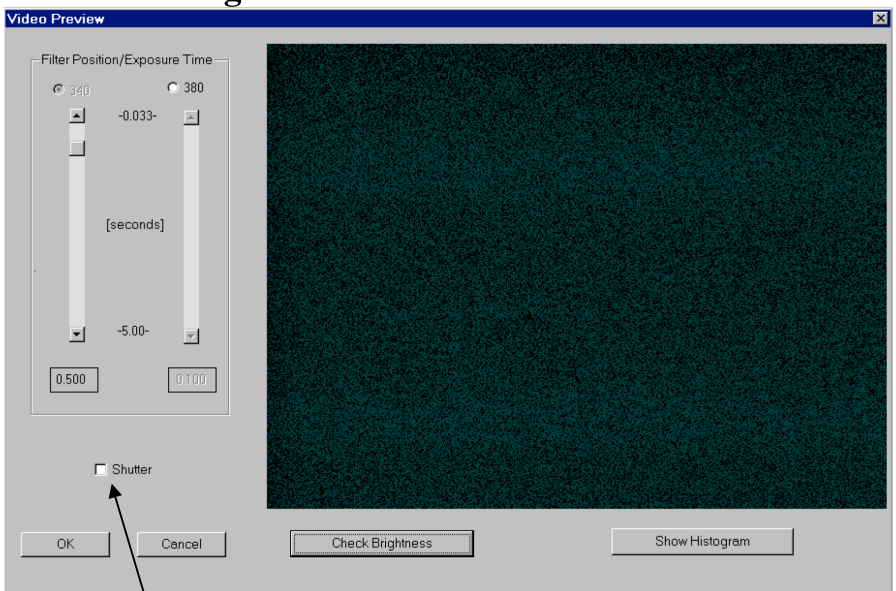
Ion labels and wavelengths appear here. To change them, go to the **<Sutter Wheel Setup>** screen.

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 **<Measure Ion During Experiment>** with the observation 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.

B. SETUP

3. Sutter Wheel Setup

a. Program notes



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

Version 4.70 for the Sutter Wheel has two setup files: 1) The InCytIm2.SET or InCytIm1.SET found in our other programs, and 2) Sutterwheel.CFG.

The Sutterwheel.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 10ms longer than that specified by Sutter (see Sutter manual), to ensure that the filters are in position when the measurement is being taken.

V. *InCyt Im2*™ PROGRAM

B. SETUP

3. 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 first 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).

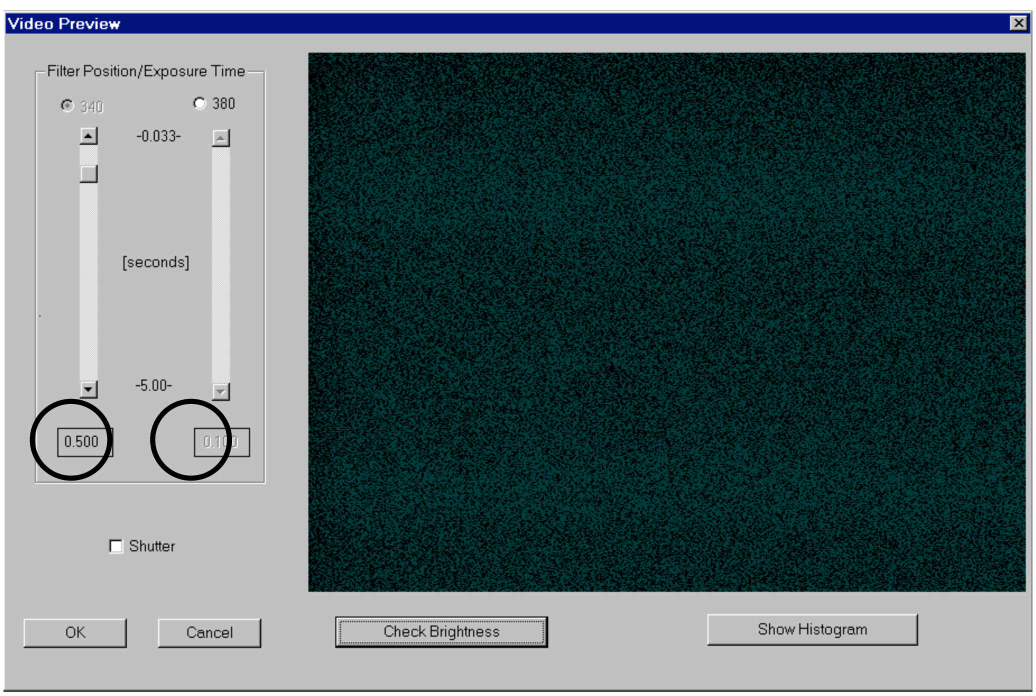
Both filter wheels allow you to change the speed setting manually. PLEASE DO NOT CHANGE THE SPEED SETTING MANUALLY WHEN USING THE INCYT PROGRAMS. THIS WILL RESULT IN A LOSS OF CPU CONTROL OF SPEED, POSSIBLY RESULTING IN INCORRECT FLUORESCENCE MEASUREMENTS. If you change the speed settings manually, please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

C. VIDEO PREVIEW

There is a detailed explanation of **<Video Preview>** in Section V.B.2.a., of this manual, "Creating a New Graph From Solutions". 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 the other wavelength. The picture is refreshed at a rate determined by the exposure time. For example, as shown below, the 340nm picture will be refreshed twice every second, while the 380nm picture will be refreshed 10 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 any 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 0.500 seconds and 380nm exposed for 0.100 seconds -- e.g., both gray-level readings are 75 (gray level readings range from 0 to 255) -- for a ratio of 1.00:1. Now if you increase the 380 exposure time to 0.200 seconds without changing the 340 exposure time and take another reading, the gray level at 380 will have increased to 150 and the new ratio will be 2.00:1. If the calibration curve were built on 340/380 exposure times of 0.500/0.100 seconds, the system would now measure Ca^{2+} concentration as much higher 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.



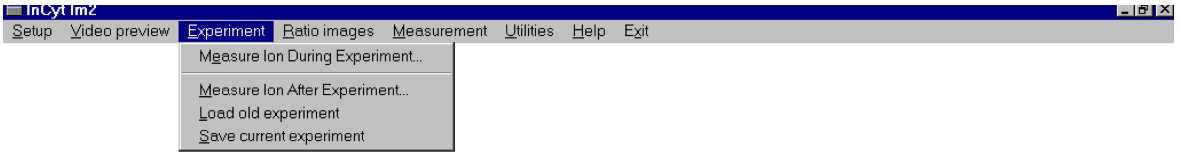
C. VIDEO PREVIEW

You can alter **BOTH** exposure times by the same factor. For example, 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 0.500 second at 340nm & 0.100 second 380nm up to 1.000 second and 0.200 second, respectively. Note that both times have been increased by a factor of two. (If you increase lamp intensity or exposure times, you should always **<Check Brightness>** at both wavelengths to make sure that the longer exposure times do not result in overexposure (as indicated by saturated, or RED, pixels).

If you find that a plate of cells is too bright and pixels are saturating, you can either lower the lamp intensity or reduce both the exposure times. Please be aware that you do not have complete flexibility in setting exposure times. All exposure times must be in multiples of 33 milliseconds. Therefore, you could not divide exposure times in half with this example. Instead you would have to reduce them by a factor of one-third, to 0.333 seconds and 0.067 seconds.

<Check Brightness>
Possible gray-level range: 0 to 255.
GREEN pixels: Gray-level = 0
RED pixels: Gray-level = 254 or 255

D. EXPERIMENT



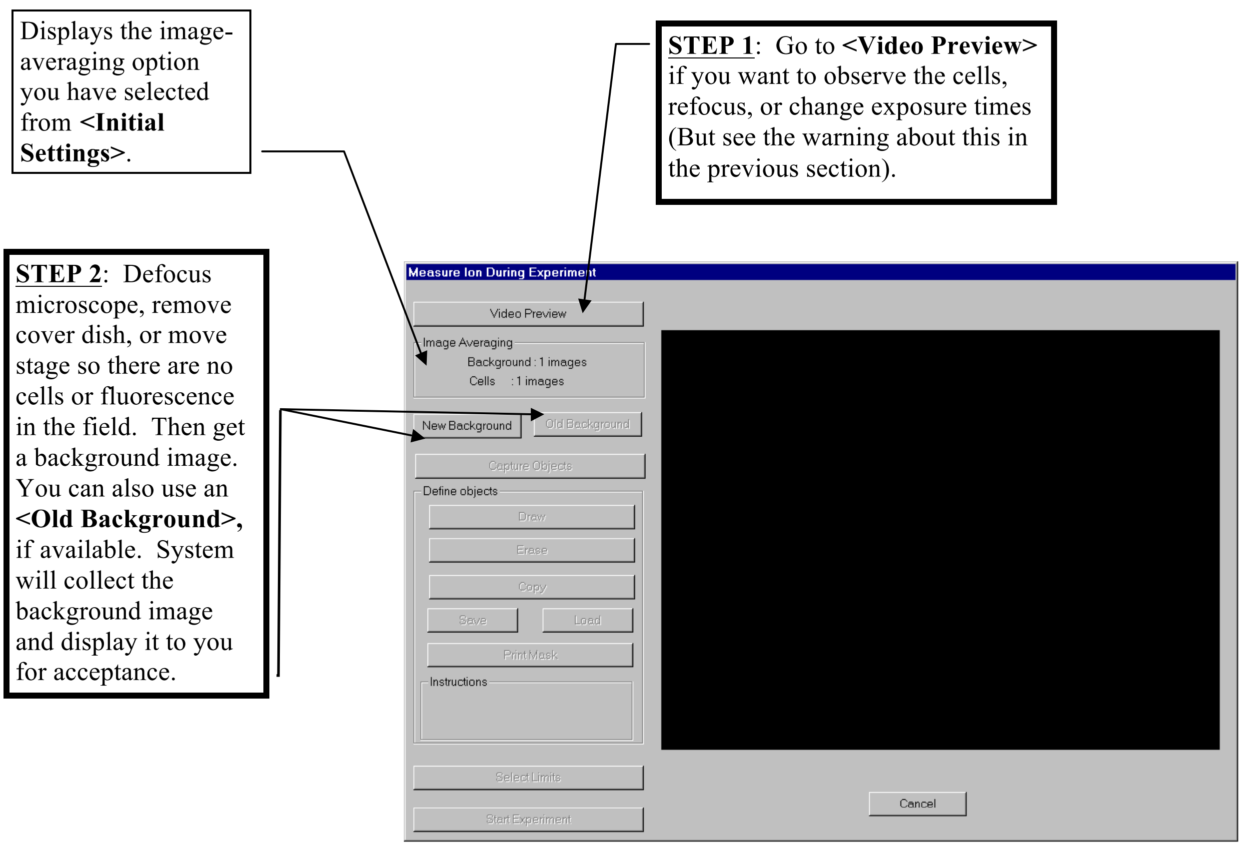
This is where you determine how you will conduct the next experiment. It is also the point from which you can load an old experiment for further analysis or save the current experiment.

1. Measure Ion During Experiment

In this method you pre-select the cells or object areas. After starting the experiment you receive immediate pictorial and graphical tracking of cell responses.

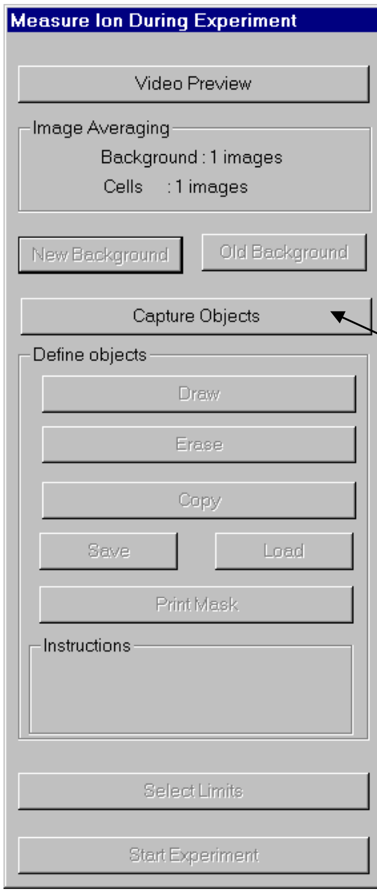
Note below that most menu items are grayed out. As you complete each section, the options below it will become available.

Select **<Measure During Experiment>** from **<Main Menu>**.



D. EXPERIMENT

1. Measure Ion During Experiment

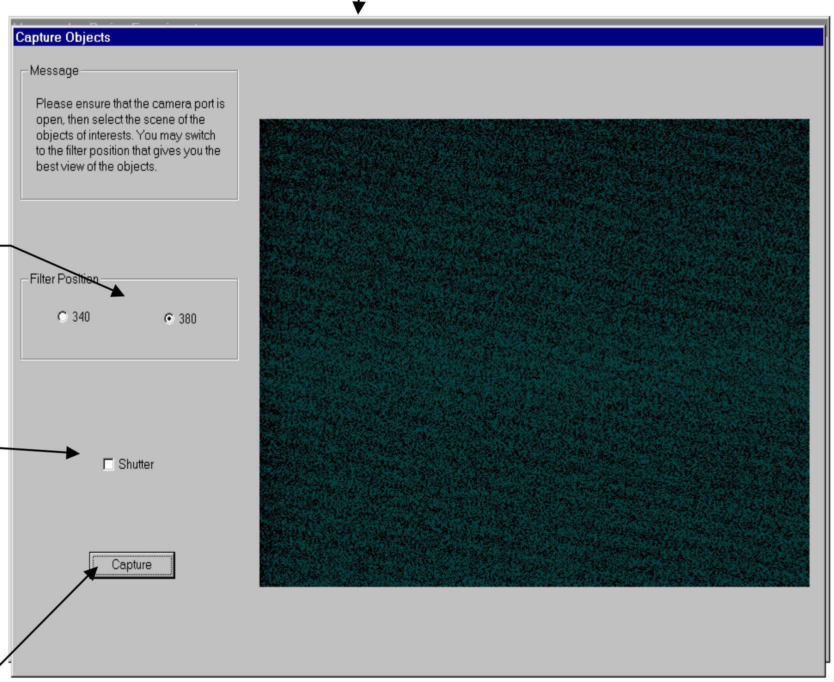


STEP 3: Click on this bar. The program sends you to the new dialog box below, which is a live video picture. Choose the wavelength to view cells and refocus.

Usually the 380nm view will be brighter when cells are at rest. Also the exposure time is likely to be shorter, so focusing will be easier.

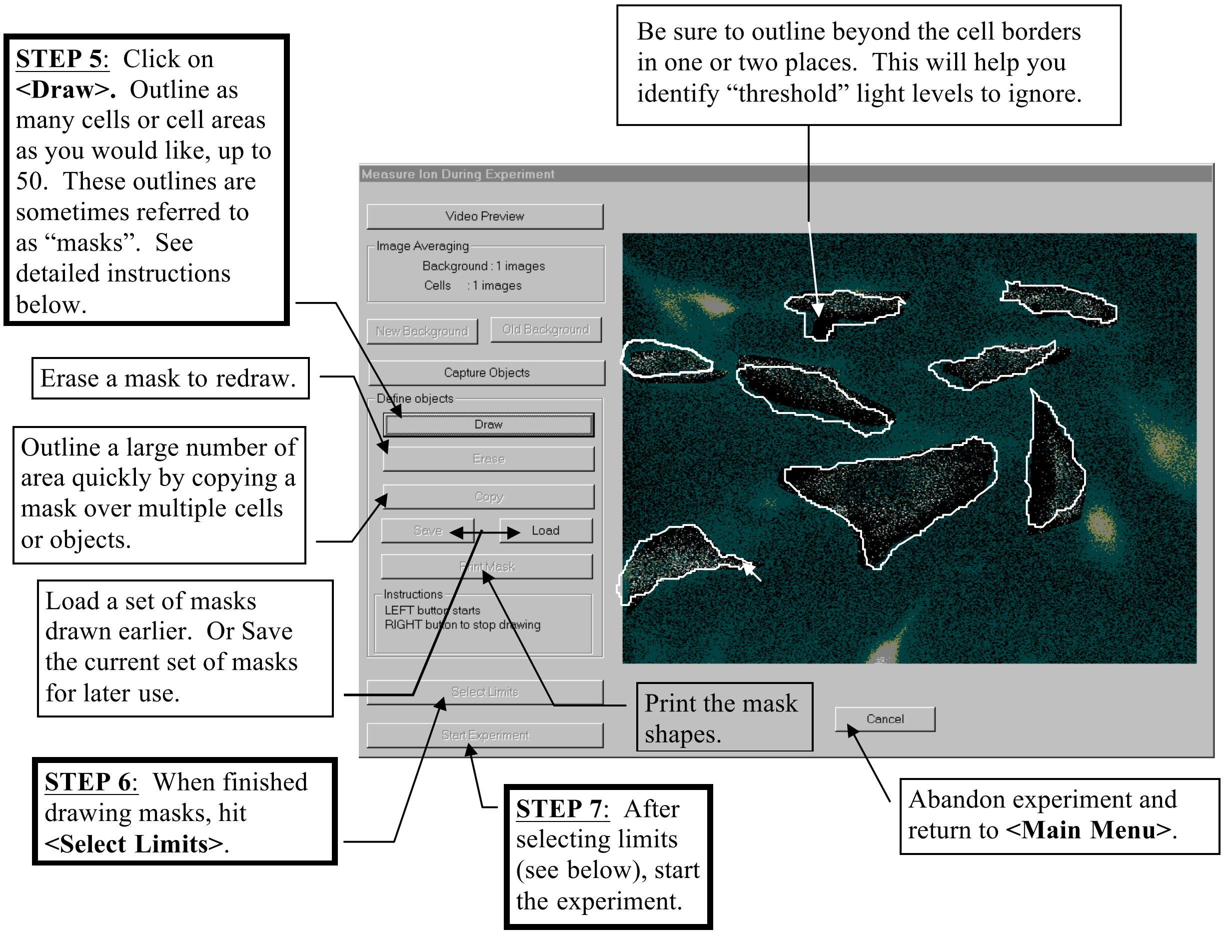
Invoke the shutter if you leave the system for any length of time to avoid bleaching and photodamage.

STEP 4: Click on this button to return to the previous screen.



D. EXPERIMENT

1. Measure Ion During Experiment



a. Drawing Instructions

When using the buttons in the Defining Objects section of this screen, use the LEFT mouse button to select buttons and execute the 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 50 areas of interest to measure.

<DRAW> After you hit the **<Draw>** 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 circle will close. You have now created a measurement "mask" for this area. To start another mask, position the cursor and hit the right mouse button again. To stop drawing, hit the RIGHT mouse button. As noted above, on one or two cell areas, extend the mask beyond the area of interest to an open area of the field of view. This extended area will be used in the next operation, **<Select Limits>**.

D. EXPERIMENT

1. Measure Ion During Experiment

a. Drawing Instructions (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 and hit the RIGHT mouse button -- and the mask disappears. Repeat for all masks you wish to eliminate or redraw. When finished erasing, hit the RIGHT mouse button.

<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. The cursor will then drag a copy of this mask as you move the mouse. When the mask copy is positioned properly, hit the LEFT mouse button again. Repeat as many times as necessary. To stop copying, hit the RIGHT mouse button.

<SAVE> If you wish to save the mask outlines that you have drawn, hit the **<Save>** button. This will prompt you to provide file name; the system will append an **".OBJ"** extension ("OBJ" or "Objects") to the file name. Saving the mask objects is useful, because you will not have to redraw the masks if 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 an **".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 relative position of each cell 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 relative position after the experiment is over. Each cell's data is marked with the object number for that cell in the data file.

D. EXPERIMENT

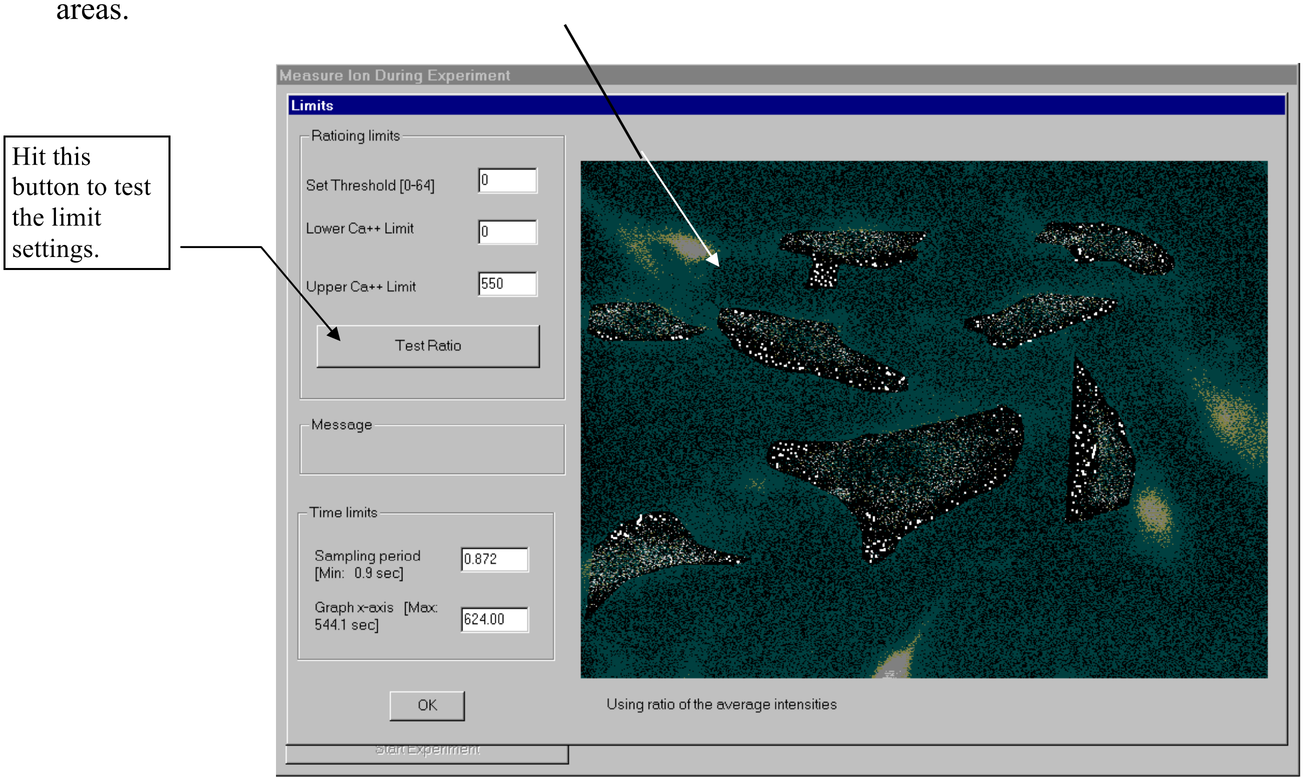
1. Measure Ion During Experiment

b. Selecting Limits for Measurement

<Set Threshold>

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 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 and will be ignored. Only cell responses above this threshold will be measured.

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, Ca^{2+} measurements will be understated. The areas you drew that were beyond the cell boundaries can be visually checked for proper thresholding. Set 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. In the screen below, the threshold is set to zero and there is quite a bit of stray fluorescence in one of the test areas.



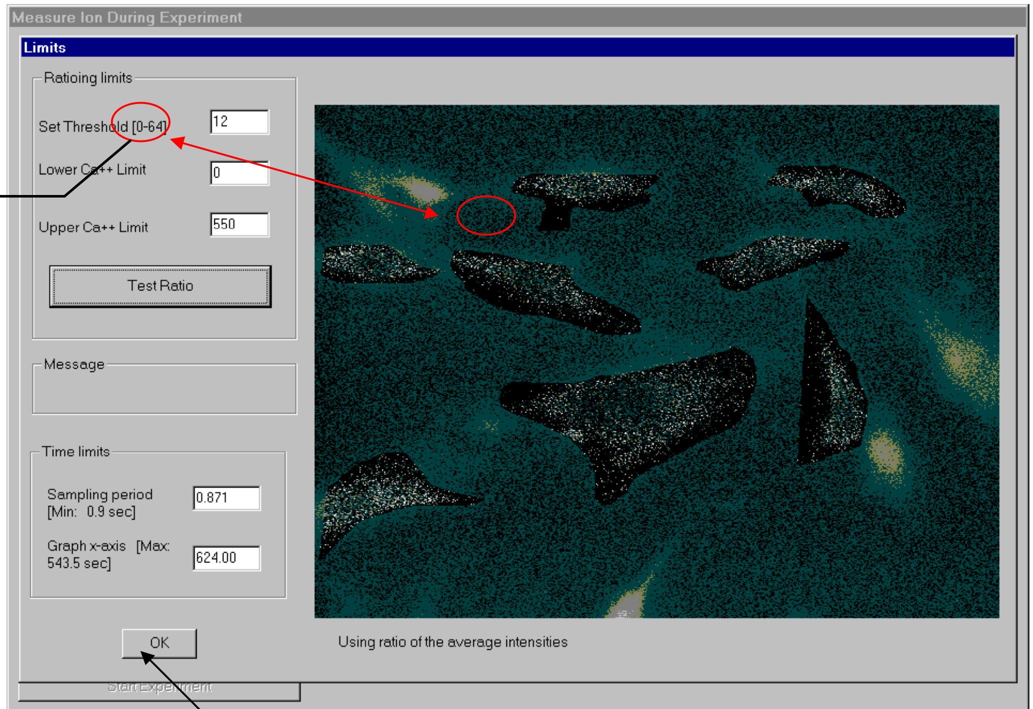
The image on the following page shows the impact of raising the threshold.

D. EXPERIMENT

1. Measure Ion During Experiment

b. Selecting Limits for Measurement (continued)

An increased threshold results in a reduction in noise.



Hit **<OK>** when done.

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

These limits affect the axis limits on the graph displayed during the experiment only -- the actual Ca^{2+} measurements are not affected. We recommend keeping the Lower limit at zero and setting the Upper limit just higher than the maximum Ca2+ level you expect to observe during the experiment.

c. Selecting Time Limits for Measurement

<Sampling Period>

This is how often a measurement will be taken by the system. The minimum sampling period, which in the above example is approximately 0.9 seconds, is calculated based on the exposure times set in **<Video Preview>** and displayed below the dialog box. 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. All numbers are in seconds.

<Graph x-axis>

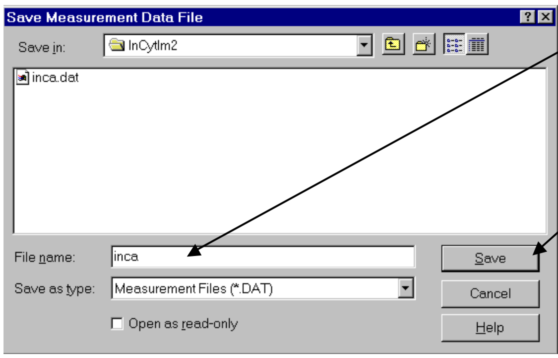
This simply shows the length of the time axis to be used for the graph displayed during the experiment. Shorten the default time if your experiment is expected to be brief.

D. EXPERIMENT

1. Measure Ion During Experiment

d. Accepting Limits for Measurement

Once you hit **<OK>** on the **<Limits>** screen to accept your settings for threshold, sampling period, and graph axis labeling, you will return to the main **<Measure During Experiment>** screen. Now hit **<Start Experiment>** to begin. You will first be prompted to create a file for the data. This file will be given a **".DAT"** extension.

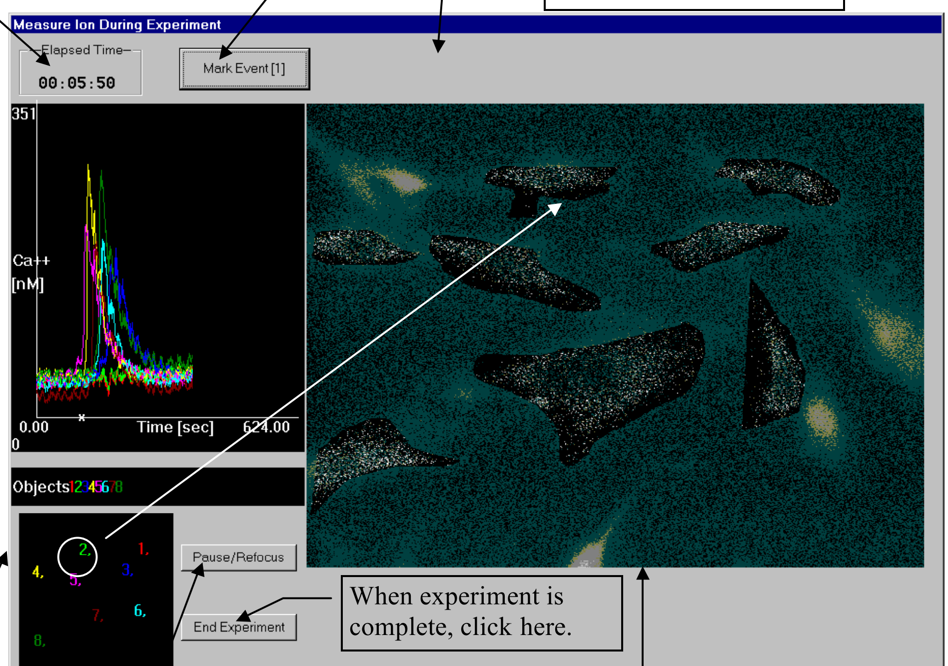


Type in a filename here. For instructions on how to access this file, see the discussion on the **<Main Menu>** item **<Measurement>**.

Hit **<Save>** to accept the file name and begin the experiment.

Mark up to 10 events during the experiment. An "x" is displayed on the x-axis below and the time is saved in the data file.

Keeps the total time of the experiment.



Calcium levels for each area of interest are tracked with a separate color.

Each area of interest is numbered and color-coded for identification during the experiment. The program scans the field pixel-by-pixel horizontally and down from the upper left. The first image it encounters is #1.

When experiment is complete, click here.

VERY IMPORTANT! If the cells move or go out of focus during the experiment, Ca^{2+} measurements will be incorrect. This button sends you back to **<Video Preview>** so you can refocus or reposition the cells inside the masks.

Images within masks are ratioed images. Outside the masks, the 380nm image is shown. Images are refreshed with every measurement.

D. EXPERIMENT

2. Measure Ion After Experiment

This method affords the user the most flexibility in analysis. The images are created and temporarily stored either in RAM or on a volatile area of the D:\ drive of the hard disk (depending on what option was chosen in **<Initial Settings>**). After the experiment is over, the user saves the images to the C:\ drive or other data storage device.

Then the images are available for multiple analysis runs. Because the images are saved, the user does not need to guess which cells or cell areas will respond. With saved images, the user can more easily identify and measure particular areas of cells that respond in an interesting way. If you anticipate publishing an experiment, choose **<Measure Ion After Experiment>** for the most flexibility in presenting the data.

The drawbacks of this method are that there is a limit to the total number of measurements that can be taken. Also, image acquisition may be slightly slower than in a **<Measure Ion During Experiment>**, because of the processing time required to handle the images.

To start the experiment, choose **<Measure Ion After Experiment>** from the **<Experiment>** menu.

D. EXPERIMENT

2. Measure Ion After Experiment

a. Running the Experiment

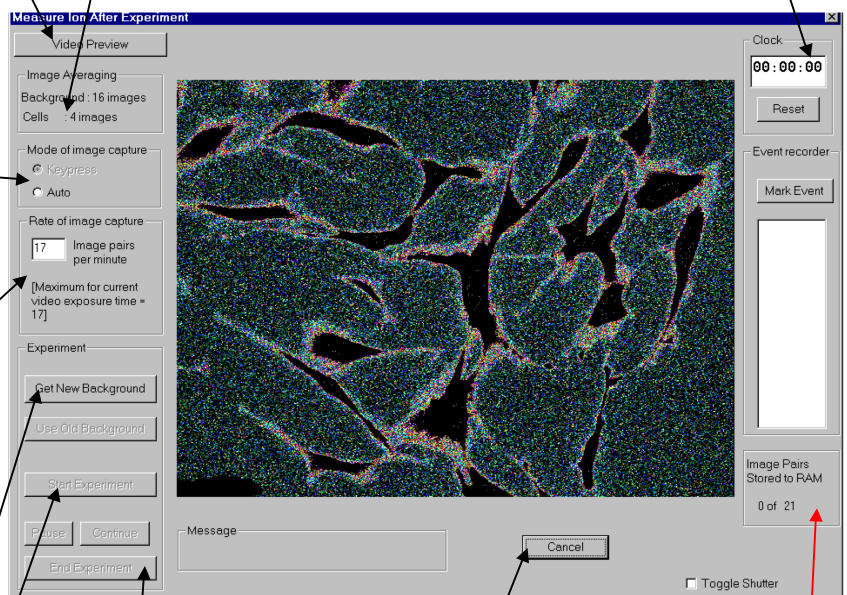
FIRST: Go to <**Video Preview**> if you need to change the wavelength for experiment observation OR if you need a "live" view to refocus or move the sample.

Image Average choices made from <**Initial Settings**>.

Keeps track of experiment time. Each image is stamped with the time.

At any time you can choose manual (keypress) or automatic image capture.

STEP 1: Choose the rate of automatic image capture. Maximum rate is calculated based on exposure times and image averaging levels.



STEP 2: Capture an image of background light. Or use an old background captured earlier.

Exits the experiment without saving settings or data.

Employ the shutter if there is a delay in starting the experiment. Protects the sample and filters.

STEP 3: Start the experiment.

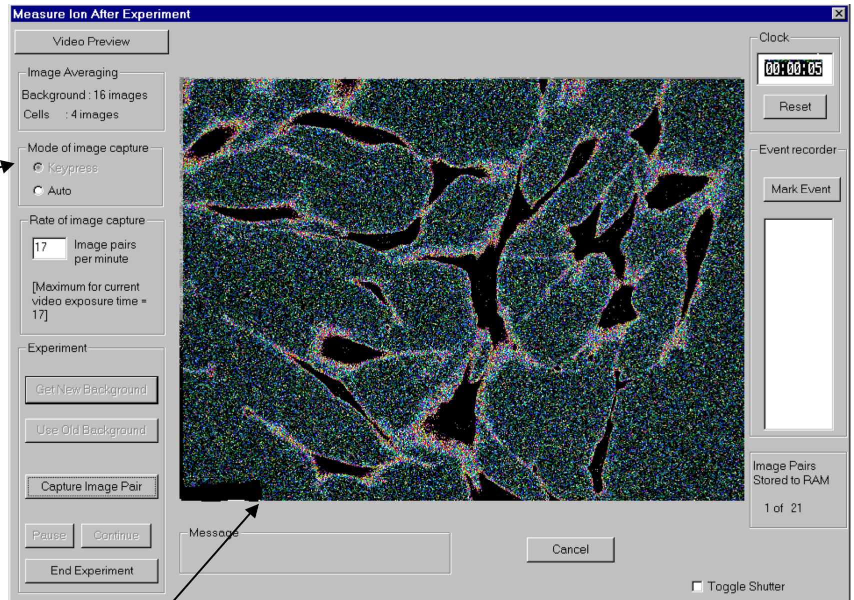
Instructions displayed after a button is pressed.

Shows image saving options chosen in <**Initial Settings**>. Especially important is the total number of images the system will collect during the experiment. If this is too low, you need to go back to <**Initial Settings**>.

D. EXPERIMENT

2. Measure Ion After Experiment

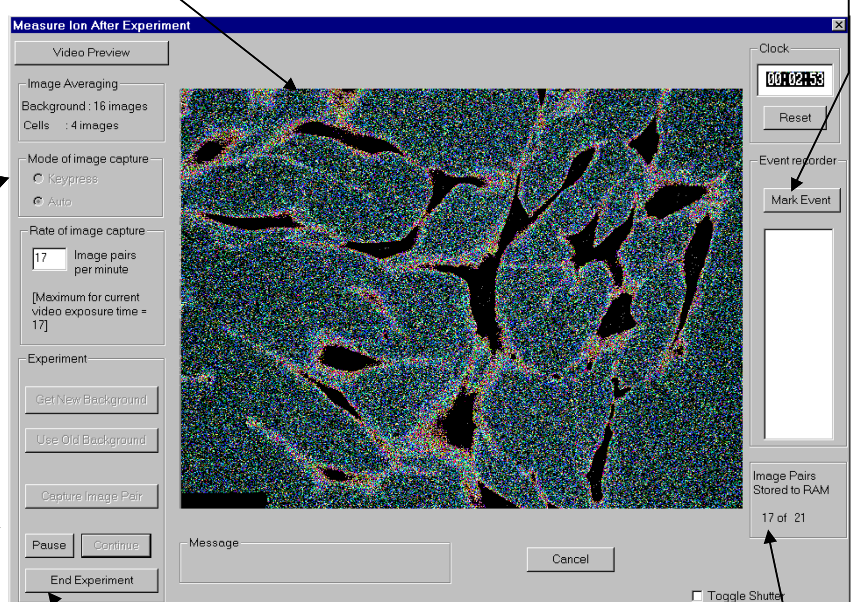
a. Running the Experiment (continued)



In **<Keypress>** mode, you are prompted to **<Capture Image Pairs>**.

The 380nm (denominator wavelength) is updated on the screen every time an image is captured.

You can "time stamp" specific events with this button. Event times appear in the box below and are captured in the data file created when the experiment is saved. The system accepts up to 10 events.



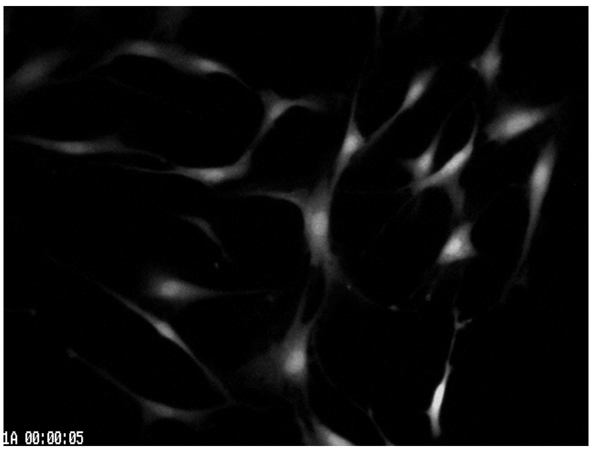
In **<Auto>** mode, you can halt image capture with the **<Pause>** key.

Click here to end the experiment.

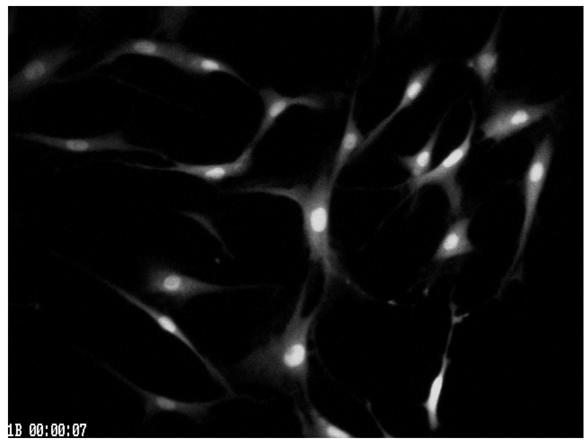
Keeps track of how many images have been taken. You can stop the experiment before the total is reached.

D. EXPERIMENT
2. Measure Ion After Experiment
a. Running the Experiment (continued)

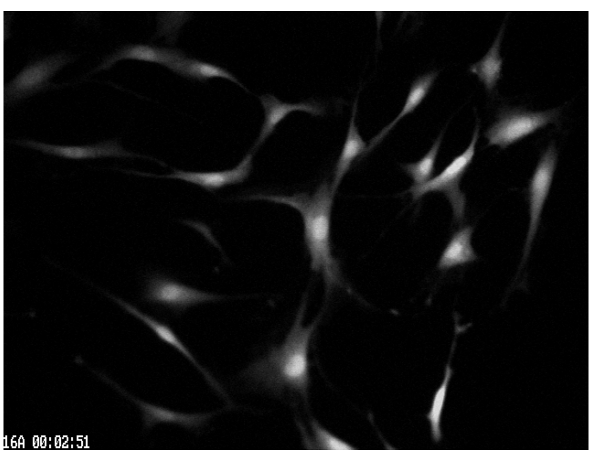
During the experiment, as Ca^{2+} levels rise, the fluorescence from the Fura-2 dye with 340nm excitation gets brighter, because more of the dye can bind to Ca^{2+} (see images to the left below). Fluorescence with 380nm excitation drops (see images to the right below). Because the change in 380nm fluorescence is greater than that of 340nm, the display provided during the experiment is the 380nm image.



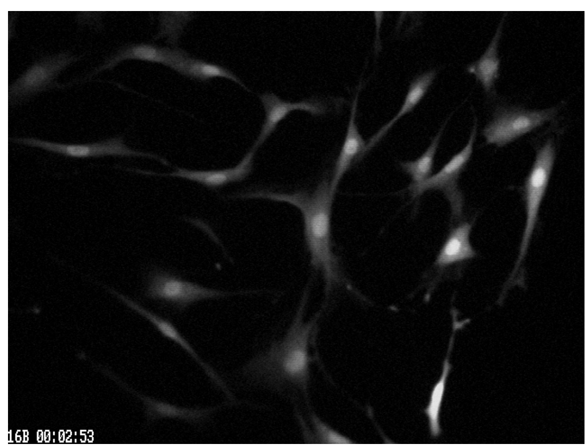
340 image of cells at rest as experiment begins.



380 image of cells at rest as experiment begins. Cells are brighter at 380 than at 340.



340 image nearly 3 minutes into experiment. Ca^{2+} levels have been rising -- the 340 images become brighter.



After 3 minutes and rising Ca2+ levels, the 380 image is markedly dimmer for those cells that are responding to the stimulus.

D. EXPERIMENT

2. Measure Ion After Experiment

b. 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”.

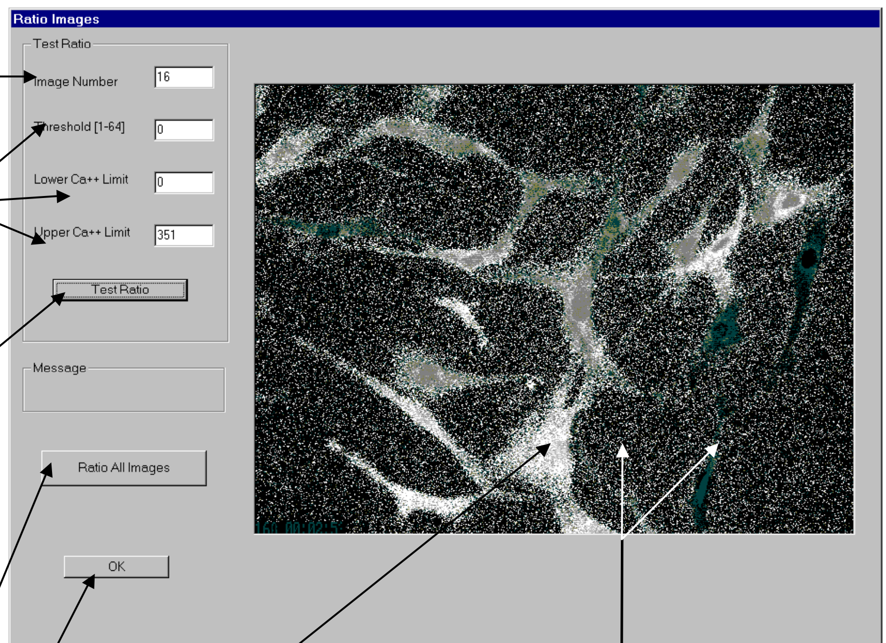
Choose any image to view while setting parameters. This image provides a good view of cells with high Ca2+ levels.

These three parameters control ratio image creation.

Test the affect of the parameters on displayed image.

Uses the above parameters to create an entire set of ratioed images. Saves these images to RAM or volatile disk memory.

Accept the above parameters and proceed to the next step, saving the experiment.



The extreme brightness of this cell indicates that the calcium level is above the **<Upper Ca^{2+} Limit>** specified above.

Notice how grainy the image is. This is low-level fluorescence noise that can be removed through thresholding. In general, there will be less noise with images that have been created with averaging.

<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 on the next page shows the impact of setting a higher **<Upper Ca++ Limit>** on the cell at the bottom of the screen.

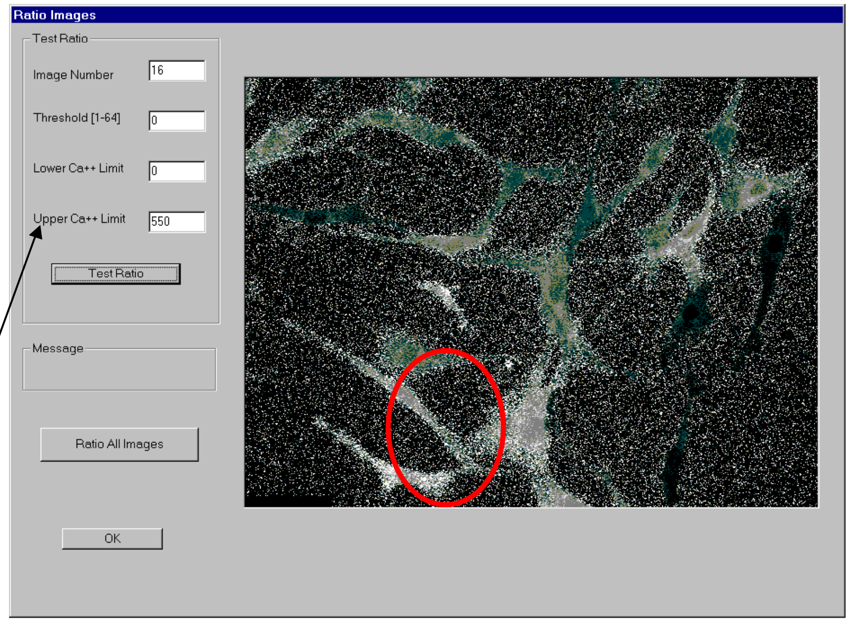
D. EXPERIMENT

2. Measure Ion After Experiment

b. Creating Ratioed Images (continued)

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.

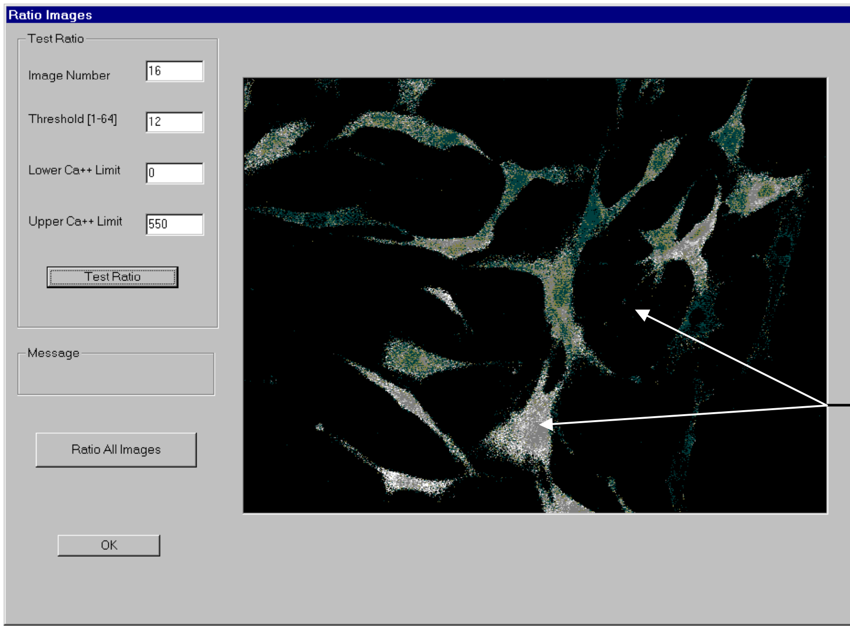
Unlike the Upper and Lower calcium limit parameters, THE THRESHOLD DOES AFFECT CALCIUM CONCENTRATION MEASUREMENTS. Only fluorescence above this threshold will be displayed and used in measuring ion concentration levels.

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 on the next page show the affect of increasing the threshold on the image.

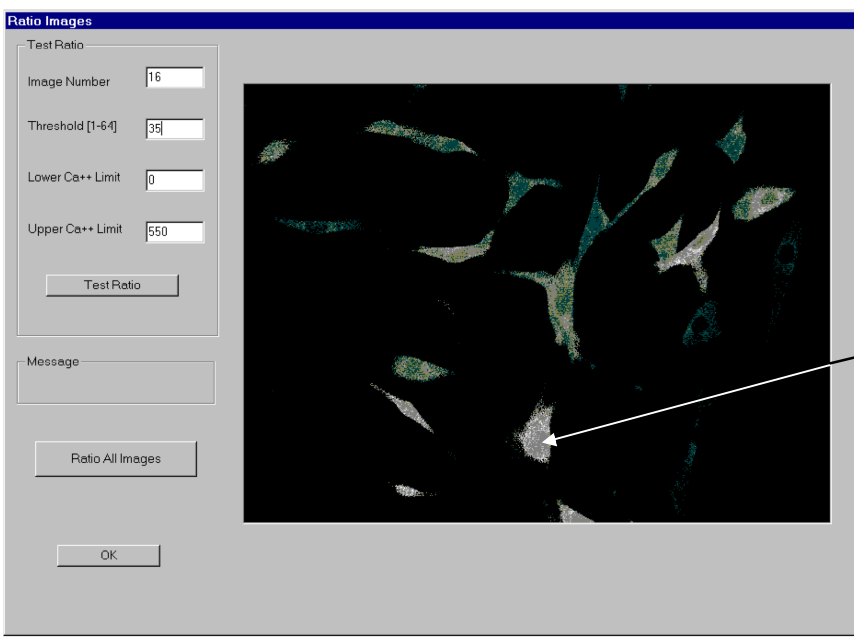
D. EXPERIMENT

2. Measure Ion After Experiment

b. Creating Ratioed Images (continued)



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

D. EXPERIMENT

2. Measure Ion After Experiment

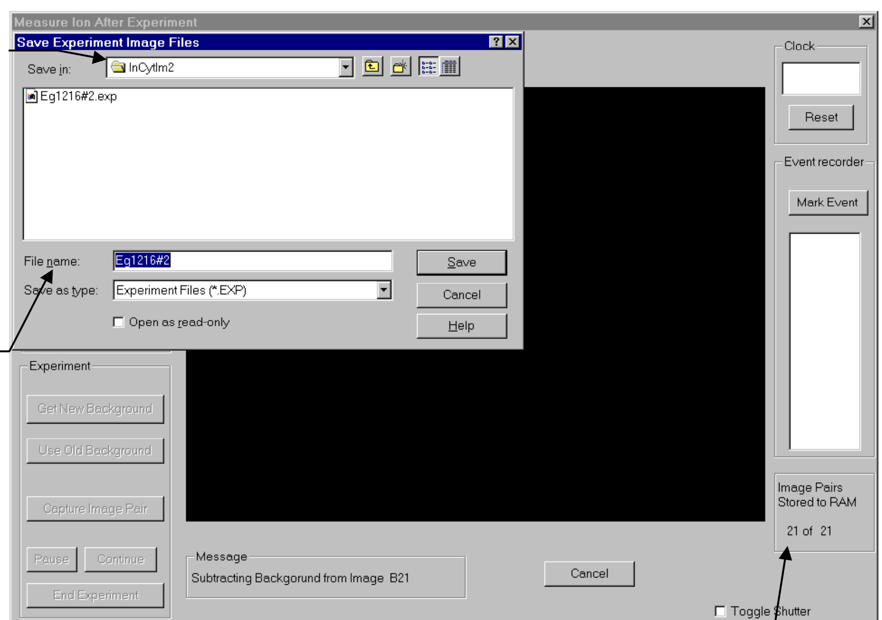
c. Saving the Experiment

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.

The first step to saving the experiment is saving the experiment file. This file contains data about the experiment -- ion measured, calibration curve, exposure times, image sizes, event time stamps, etc. It is the file called up when reloading the experiment for further analysis.

Save the experiment file to the C:\ drive or other data storage device. **DO NOT** save to the D:\ drive, unless you are using 4.7 or higher of this software.

You will be prompted for a file name for the experiment file. This file will be given an **".exp"** extension. It contains data about the experiment, but no images files.



All images have been captured.

D. EXPERIMENT

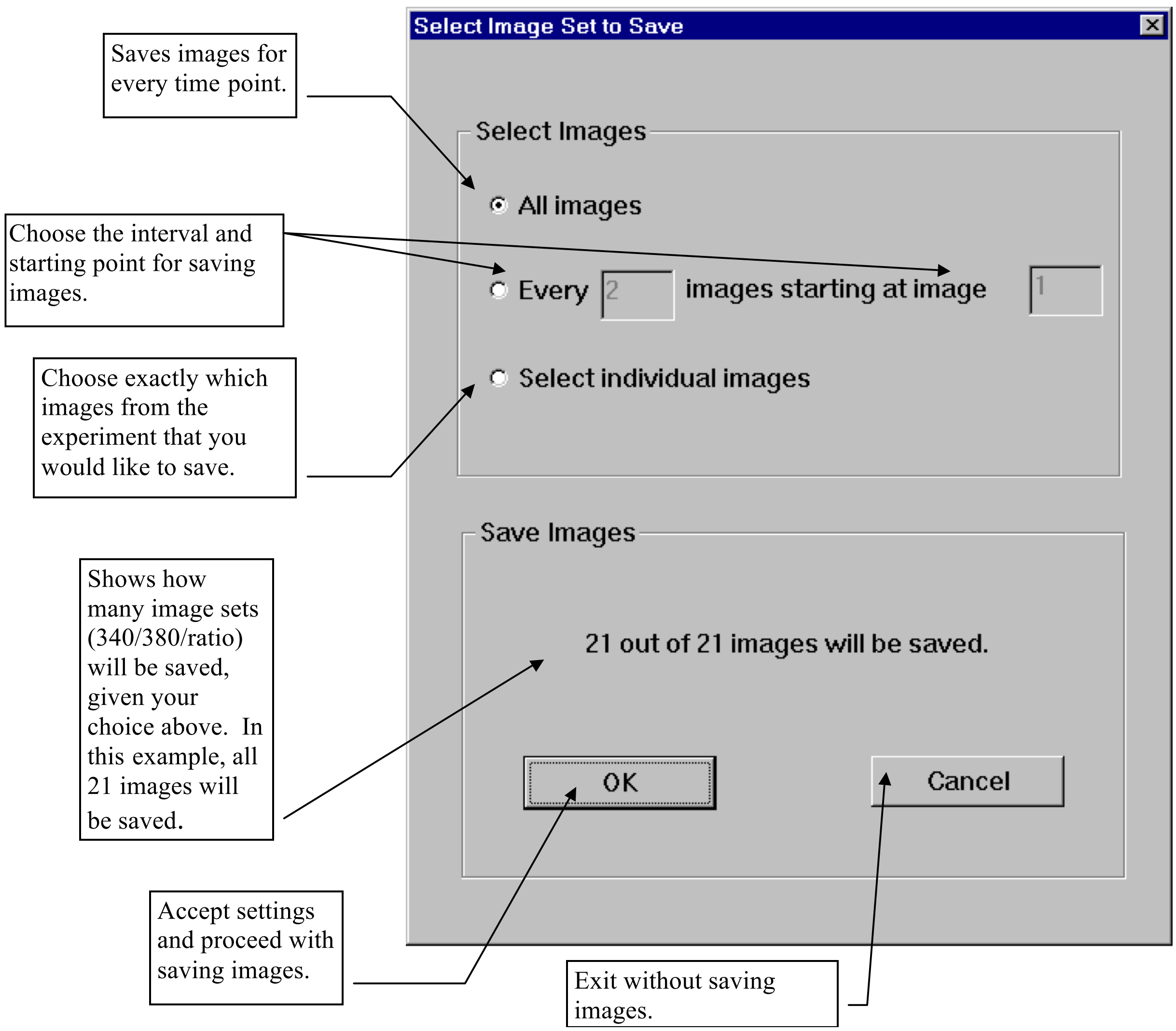
2. Measure Ion After Experiment

c. Saving the Experiment (continued)

After the experiment (**.exp**) file is saved, you are given the opportunity to save the experiment images. Images will be saved to the same drive and subdirectory as the experiment file.

NOTE: Even if you chose the <Save Images to Disk> option in <Initial Settings>, you still need to save the images permanently. During the experiment, the images are saved in a volatile section of the D:\ drive reserved for active experiments.

The first step is deciding for which time points you want to save images.



D. EXPERIMENT

2. Measure Ion After Experiment

c. Saving the Experiment (continued)

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

Select the wavelength (340/380/Ratio) that will best help you distinguish and select the images you want to save. This is for display purposes only.

Move among frames with these buttons

Hit <**Save**> to select images and <**Do Not Save**> to deselect.

Choose whether or not to add color to the images as a selection aid (see <**Pseudocolor**> discussion under <**Utilities**>). This is for display purposes only.

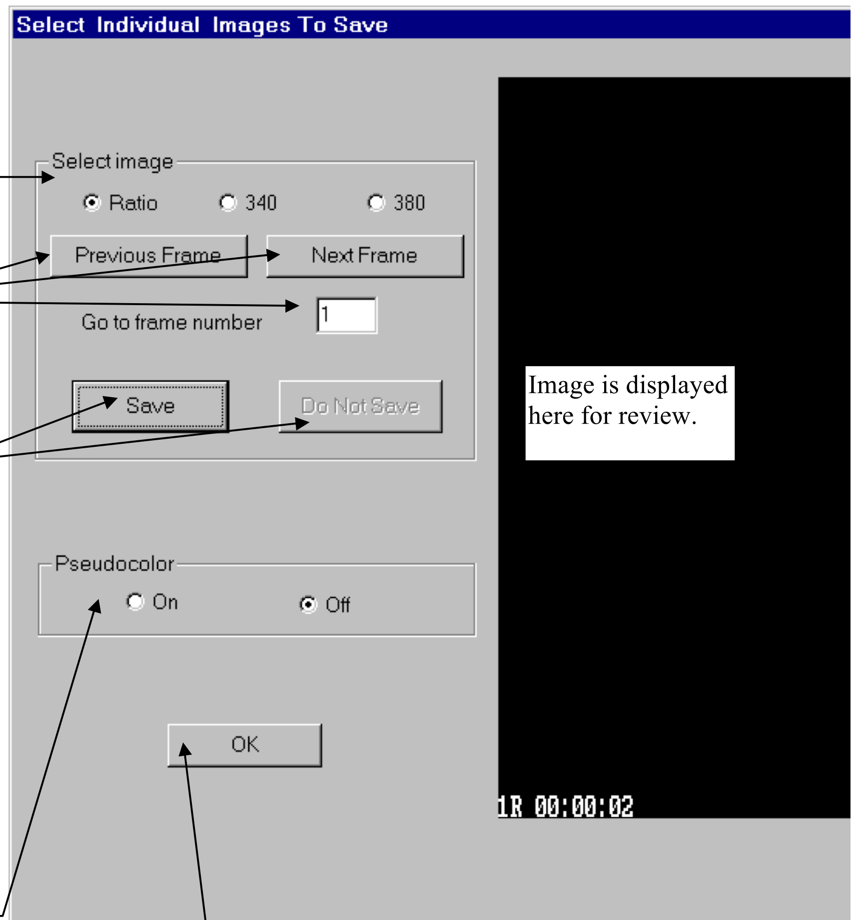


Image is displayed here for review.

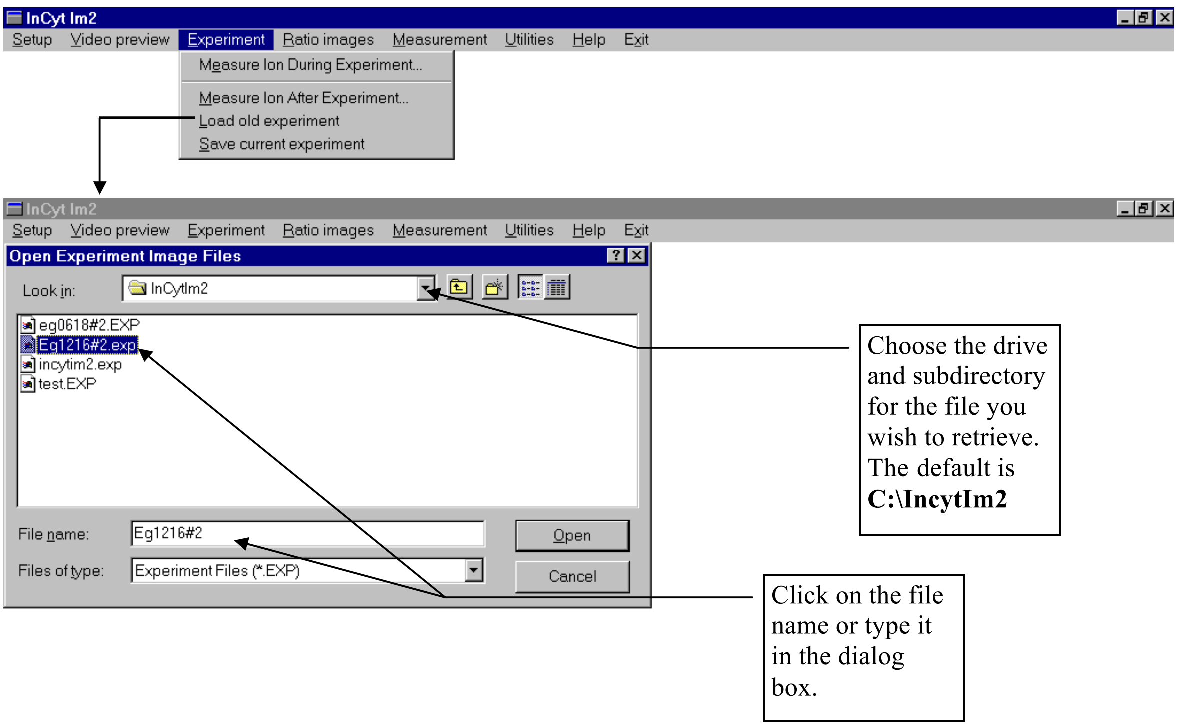
Hit **<OK>** when finished selecting images. This returns you to the previous screen, where you hit **<OK>** again to save images.

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

D. EXPERIMENT

3. Load Experiment

You can load old experiments for further analysis if they were conducted in **<Measure Ion After Experiment>** mode. From the **<Experiment>** menu, choose **<Load Experiment>**. The system will search for experiment files with a "**.exp**" extension.



You will then be prompted separately about loading each series of images from the experiment -- 340nm, 380nm, and ratio.

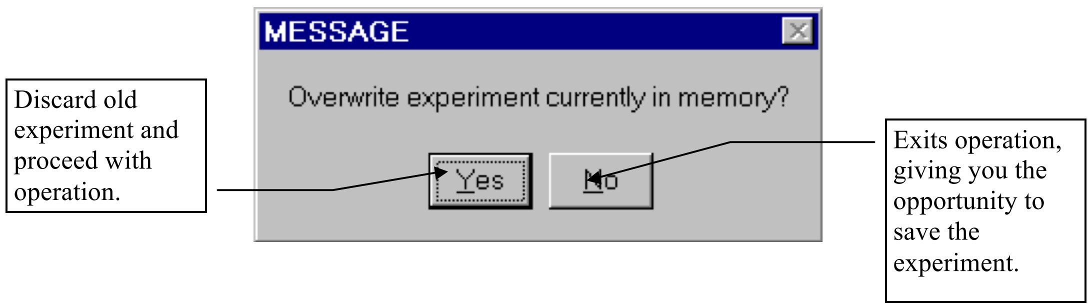
NOTE: The system assumes that the image files and the experiment (.exp) files are in the same subdirectory. If they are not, the system will return error messages. When archiving experiments, keep these files together. Image files have an extension ".TIF". The filename begins with the experiment name; then it has the character designating the image series -- "A" (for 340 or numerator wavelength), "B" (for 380 or denominator wavelength), or "R" (for ratio); finally there is a sequential number, 1 through 256 designating the image number.

D. EXPERIMENT

4. Save Current Experiment

If you did not save an experiment conducted in **<Measure Ion After Experiment>** mode immediately after the experiment, it is important that you save the experiment before starting another experiment, loading an old experiment, or exiting the program. Images for these experiments are saved in volatile RAM or hard disk space on the D:\ drive and will be lost if not saved to the C:\ drive or other data storage device.

If you fail to save an experiment and execute an operation that would delete the experiment, the system prompts you with the following message:



If you load an old experiment and change the parameters of the ratio images by using the **<Ratio Images>** utility, you will need to resave the experiment to save these images. If you resave the experiment under the same name, the old ratio images will be overwritten. You can retain both sets of ratio images by resaving to a different file name.

To save an experiment, choose **<Save Current Experiment>** from the **<Experiment>** menu. Refer to section V.C.2.c., in the **<Measure Ion During Experiment>** instructions.

E. RATIO IMAGES

You can re-ratio an experiment's images at any time.

<u>**Step 1**</u>: Load the experiment you want to re-ratio. Be sure to load all images. 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.

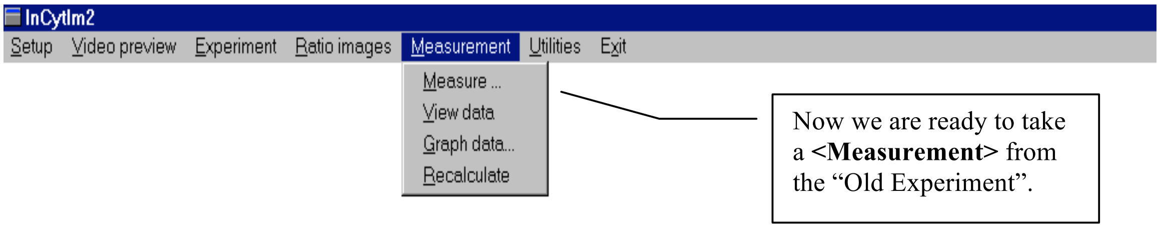
<u>**Step 2**</u>: Choose **<Ratio Images>** from the **<Main Menu>**.

<u>**Step 3**</u>: Ratio images as described in Section V.C.2.b. When you find the correct parameters, be sure to hit the **<Ratio All Images>** button.

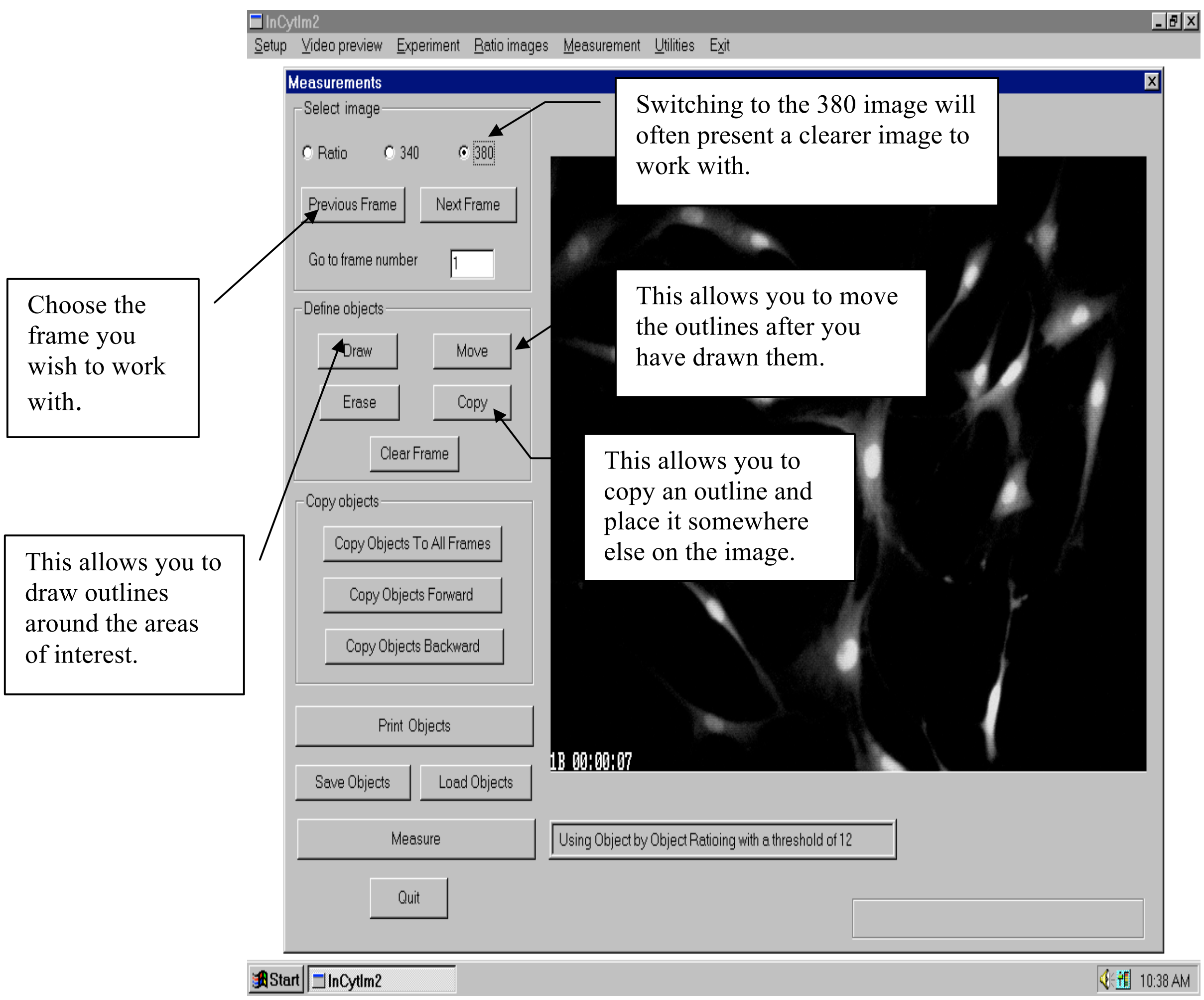
<u>**Step 4**</u>: Resave the experiment using **<Save Current Experiment>** from the **<Experiment>** menu as described in Section V.C.4. There is no need to resave the 340nm and 380nm images, but you must save the new ratio images. Again, you may save the experiment under a different name if you want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

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 outlines if the slide should get moved at some point during the experiment.

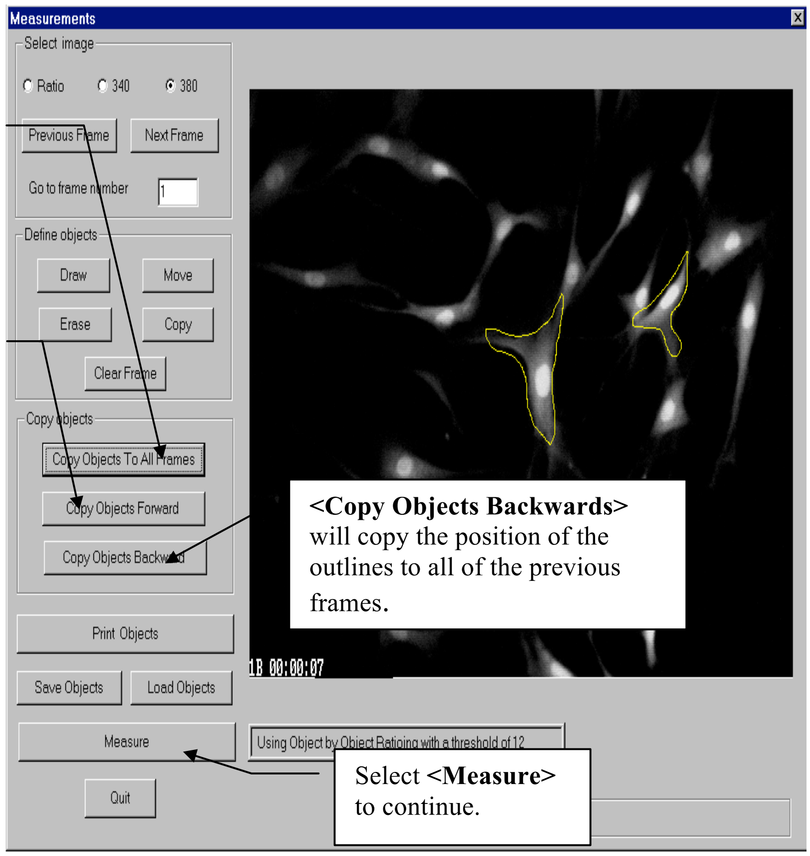


F. MEASURING THE DATA

1. Measurement

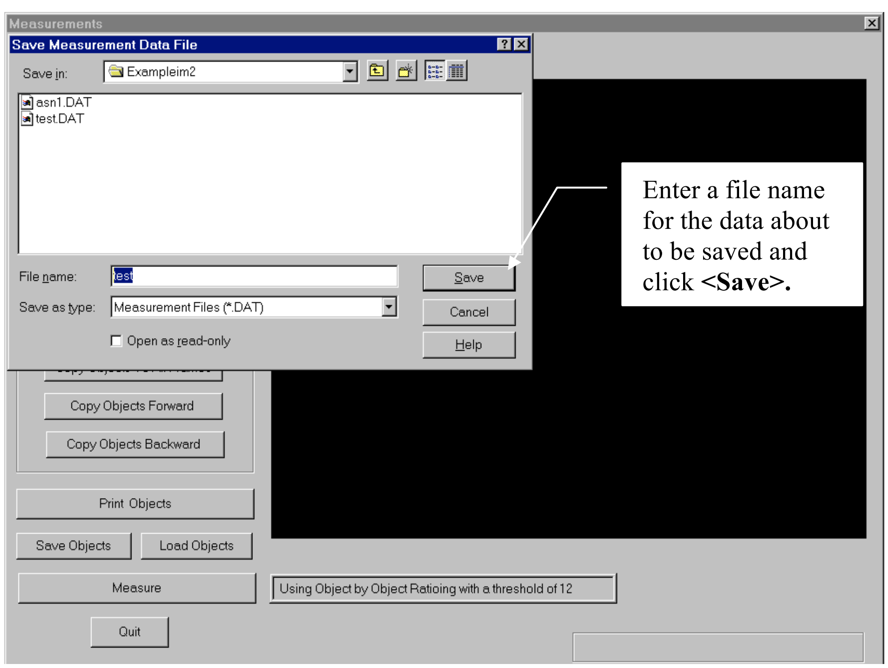
After you trace the images, remember to copy the objects to all of the frames.

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.

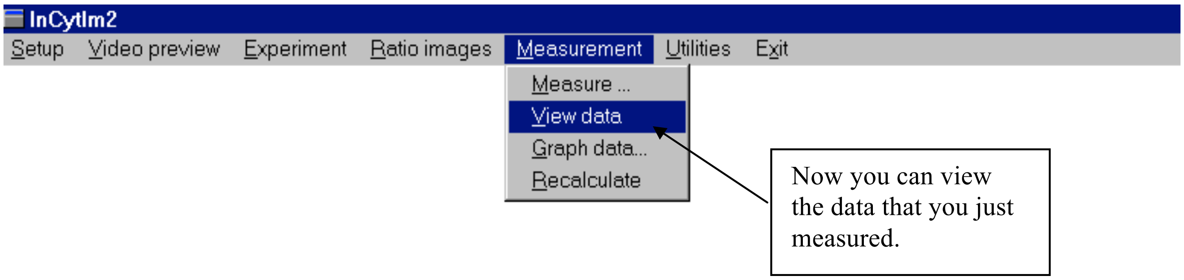
Select <**Measure**> to continue.



Enter a file name for the data about to be saved and click <**Save**>.

F. MEASURING THE DATA

2. View Data



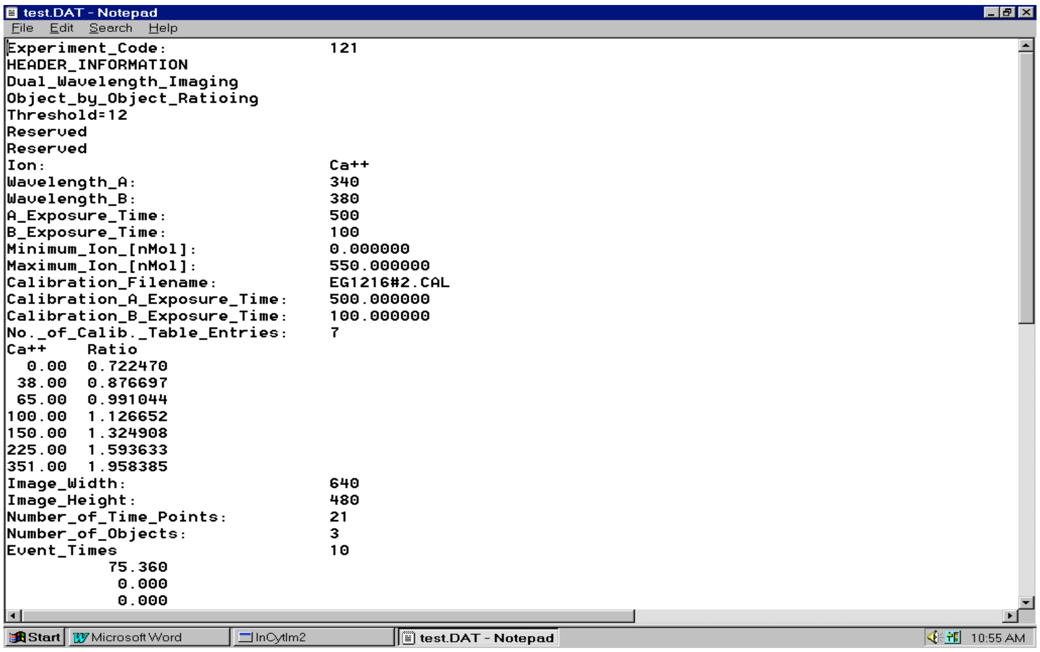
The data will be presented in notepad form. The file that the data is saved in is a tab-delimited ASCII file, which can easily be transferred to almost any spreadsheet program. The data that is presented in the heading includes the ion being measured, the wavelengths it is being observed at, and the 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

F. MEASURING THE DATA

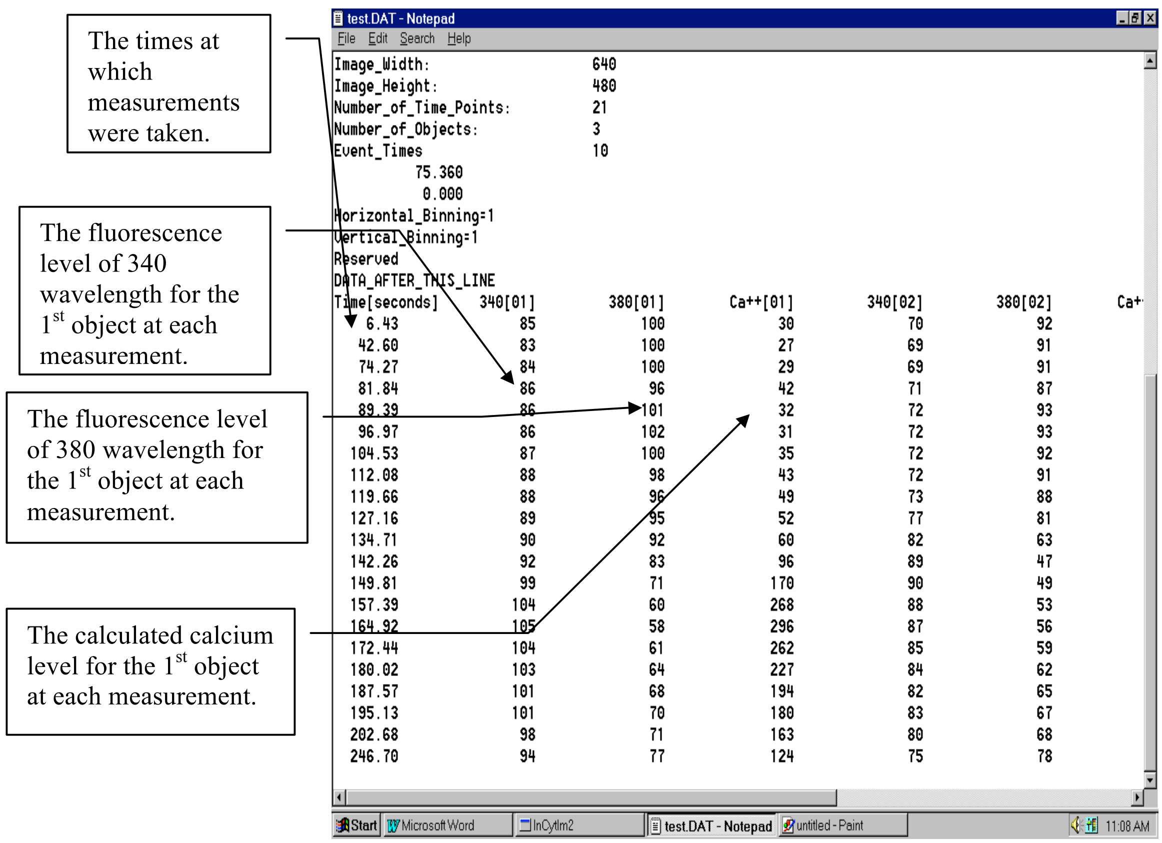
2. View Data

a. Header



```
Experiment_Code:                  121
HEADER_INFORMATION
Dual_Wavelength_Imaging
Object_by_Object_Ratioing
Threshold=12
Reserved
Reserved
Ion:                              Ca++
Wavelength_A:                     340
Wavelength_B:                     380
A_Exposure_Time:                  500
B_Exposure_Time:                  100
Minimum_Ion_[nMol]:               0.000000
Maximum_Ion_[nMol]:               550.000000
Calibration_Filename:             EG1216#2.CAL
Calibration_A_Exposure_Time:      500.000000
Calibration_B_Exposure_Time:      100.000000
No._of_Calib._Table_Entries:      7
Ca++     Ratio
  0.00   0.722470
 38.00   0.876697
 65.00   0.991044
100.00   1.126652
150.00   1.324908
225.00   1.593633
351.00   1.958385
Image_Width:                      640
Image_Height:                     480
Number_of_Time_Points:            21
Number_of_Objects:                3
Event_Times                       10
          75.360
           0.000
           0.000
```

b. Experimental Data



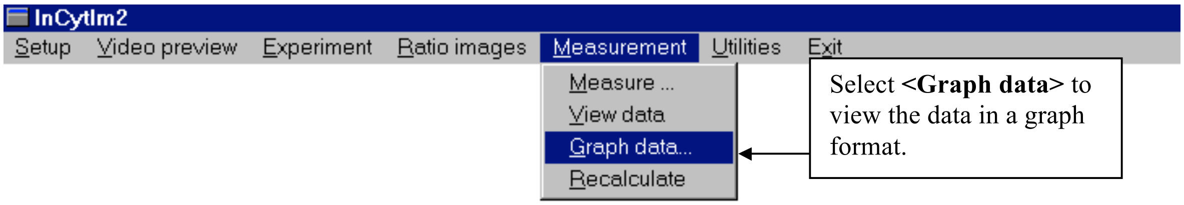

```
Image_Width:                 640
Image_Height:                480
Number_of_Time_Points:       21
Number_of_Objects:           3
Event_Times                  10
        75.360
         0.000
Horizontal_Binning=1
Vertical_Binning=1
Reserved
DATA_AFTER_THIS_LINE
```

Time[seconds]	340[01]	380[01]	Ca++[01]	340[02]	380[02]	Ca+
6.43	85	100	30	70	92	
42.60	83	100	27	69	91	
74.27	84	100	29	69	91	
81.84	86	96	42	71	87	
89.39	86	101	32	72	93	
96.97	86	102	31	72	93	
104.53	87	100	35	72	92	
112.08	88	98	43	72	91	
119.66	88	96	49	73	88	
127.16	89	95	52	77	81	
134.71	90	92	60	82	63	
142.26	92	83	96	89	47	
149.81	99	71	170	90	49	
157.39	104	60	268	88	53	
164.92	105	58	296	87	56	
172.44	104	61	262	85	59	
180.02	103	64	227	84	62	
187.57	101	68	194	82	65	
195.13	101	70	180	83	67	
202.68	98	71	163	80	68	
246.70	94	77	124	75	78	

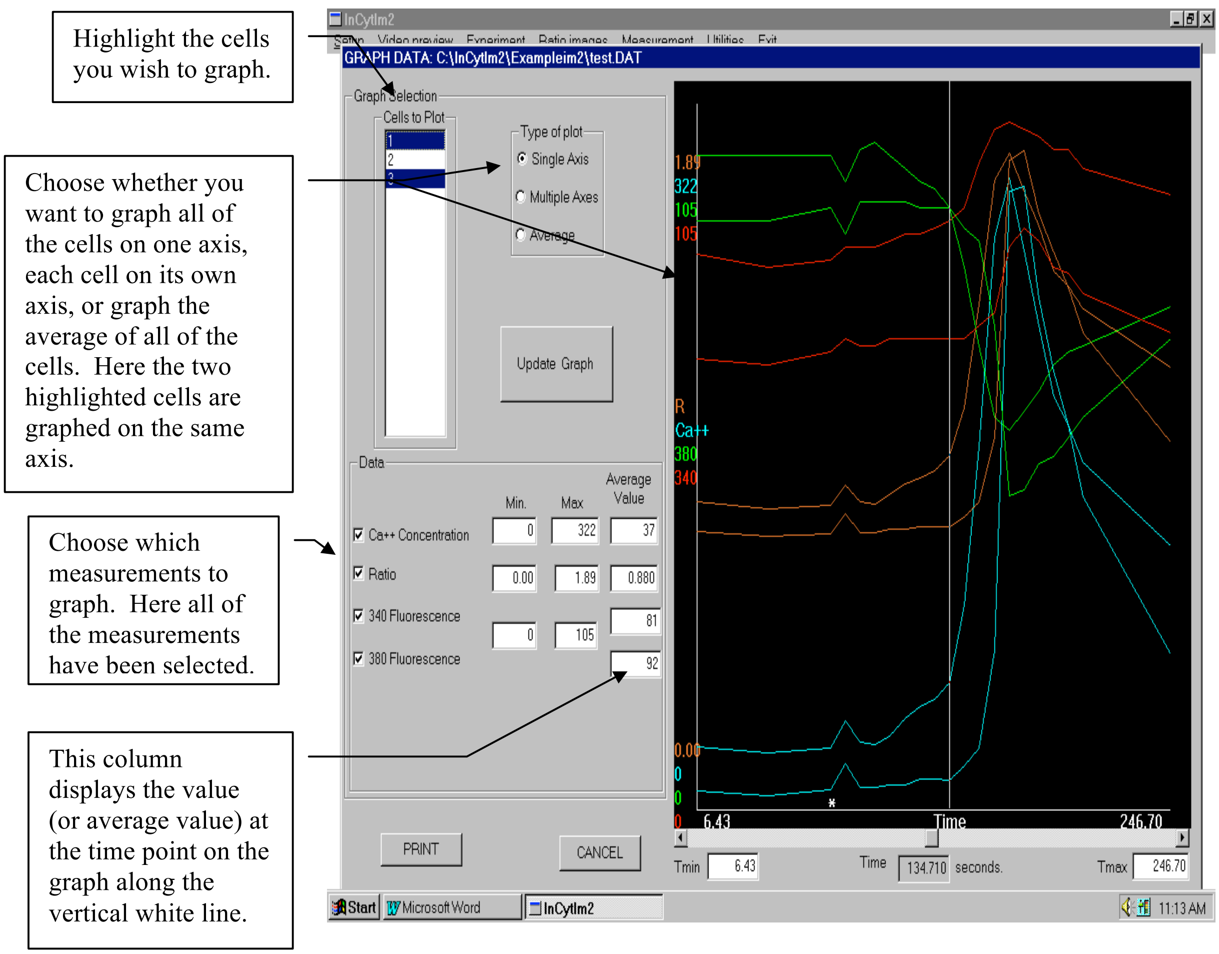
F. MEASURING THE DATA

3. Graph Data

a. Graphing Options

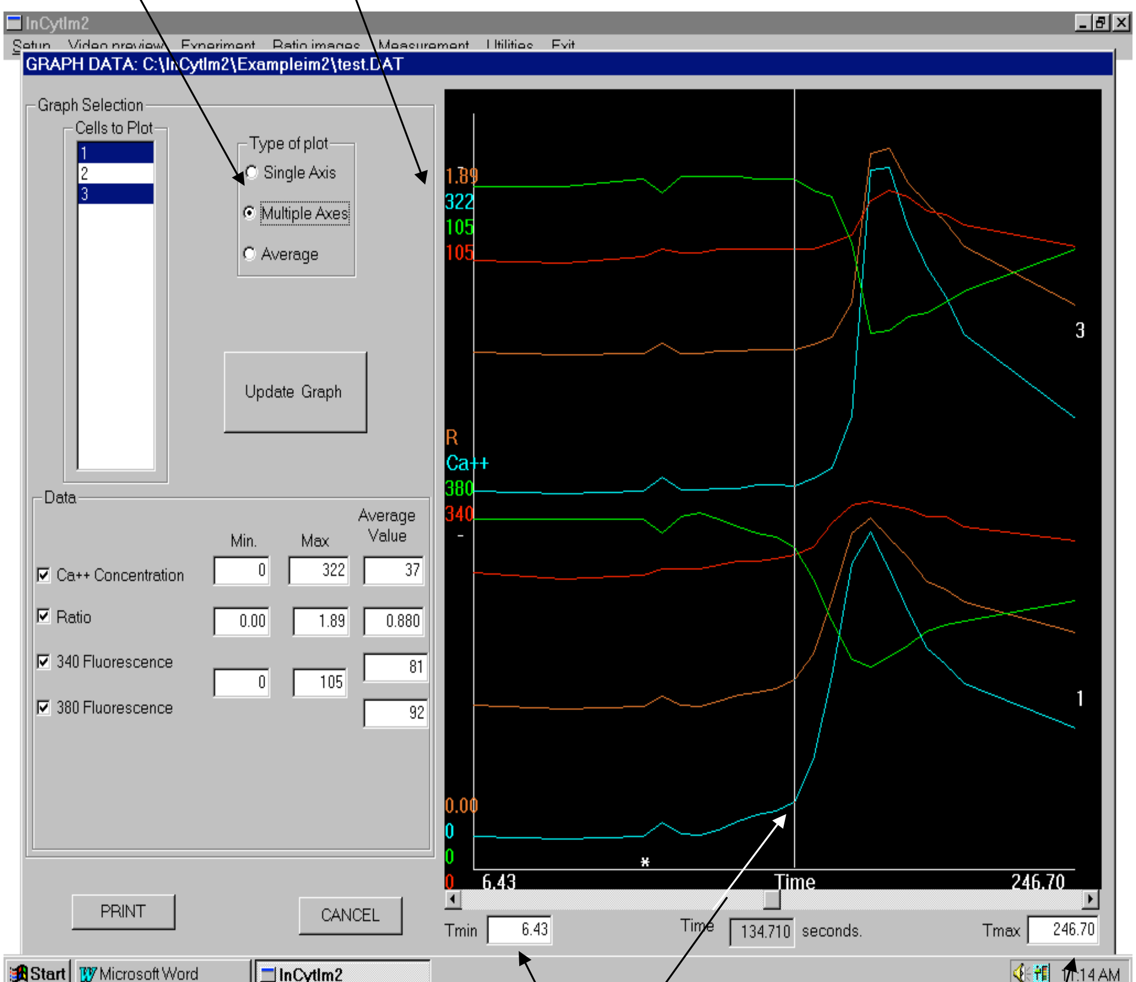


When you graph your data, you have several different options. You can graph any or all of the cells that you collected data from during the experiment. You can graph them all on the same axis, each cell on its own axis, or you can average the measurements of all of the selected cells and graph the average. 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.



F. MEASURING THE DATA
3. Graph Data
b. Timing

In this graph each of the cells is graphed on an individual axis. Object 3 is graphed above object 1.



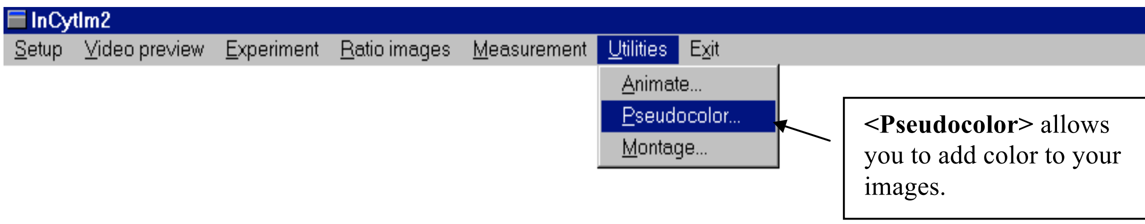
This vertical line allows you to determine the exact time of a measurement on the graph.

By changing these times you can focus in on the graph for a certain time period of the experiment or view the graph for the whole experiment.

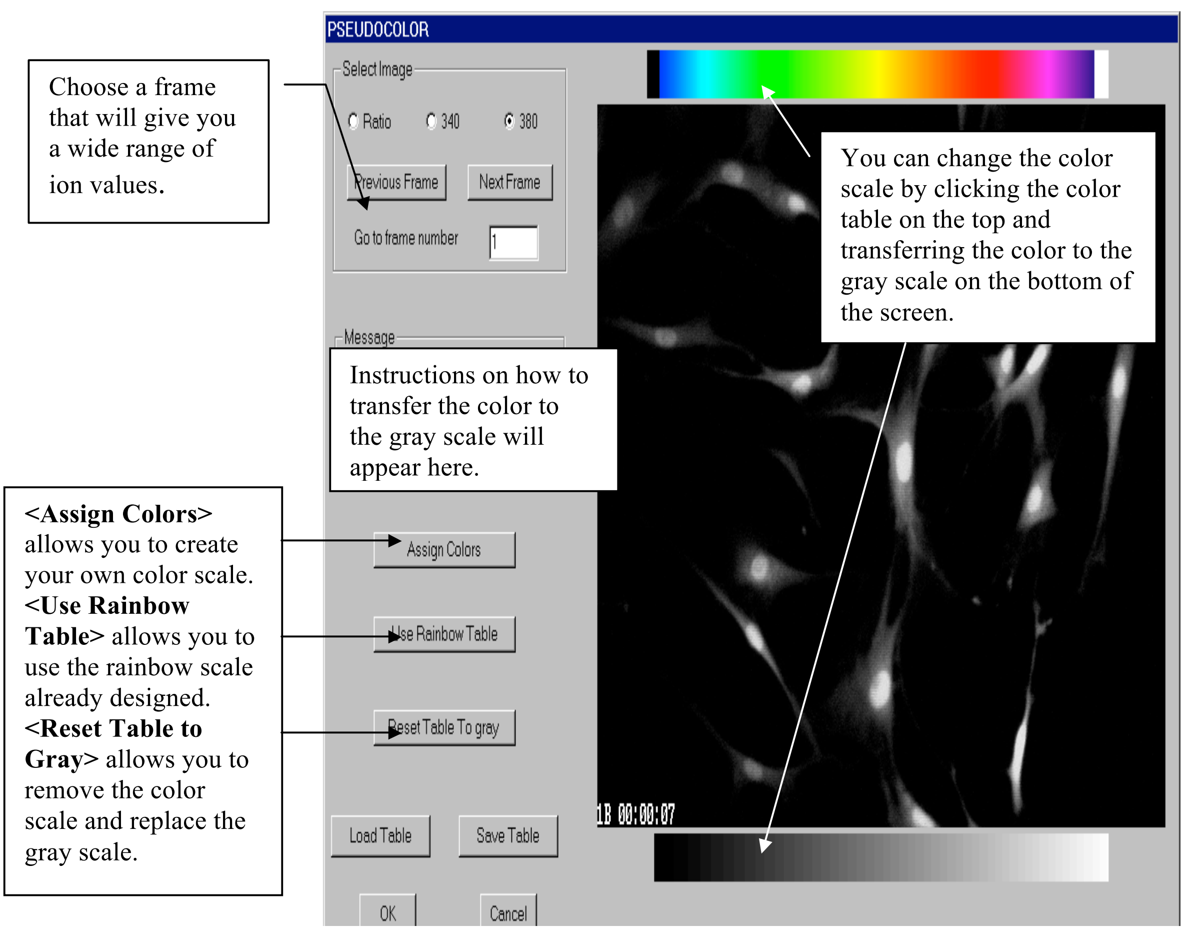
V. *InCyt Im2*™ PROGRAM

G. UTILITIES

1. Pseudocolor

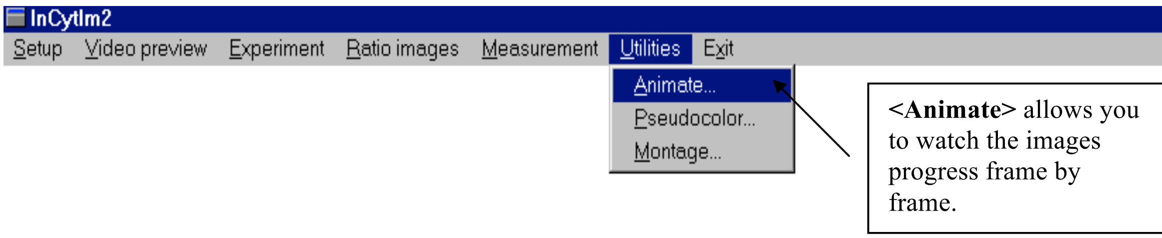


Because color changes can be easier to detect than gray scale changes, you may want to change the gray scale to a color scale. The **<Pseudocolor>** option allows you to do this. In the gray scale, the lowest levels of calcium are darkest, and the highest levels are lightest. In the typical rainbow scale, 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.

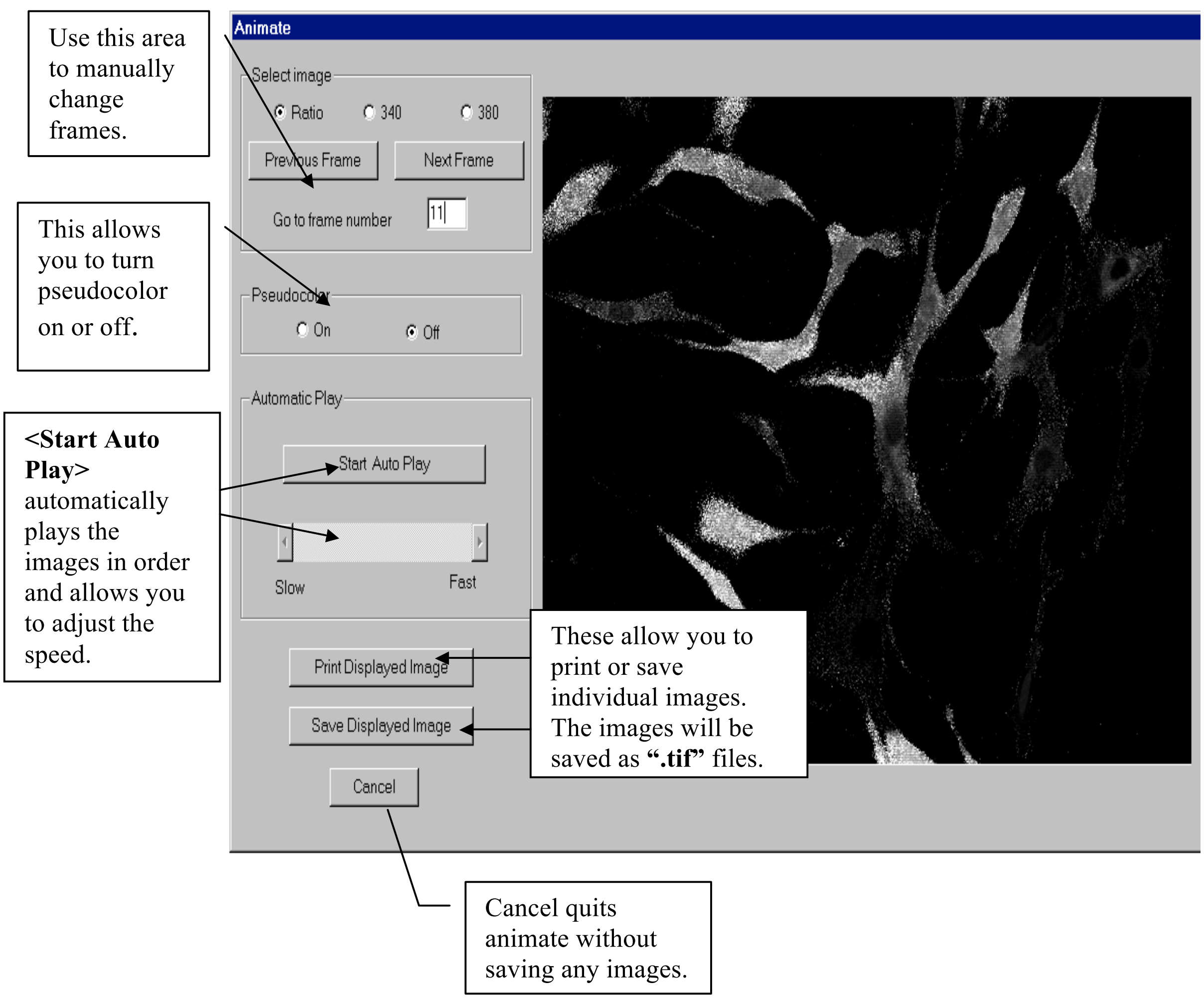


G. UTILITIES

2. Animate



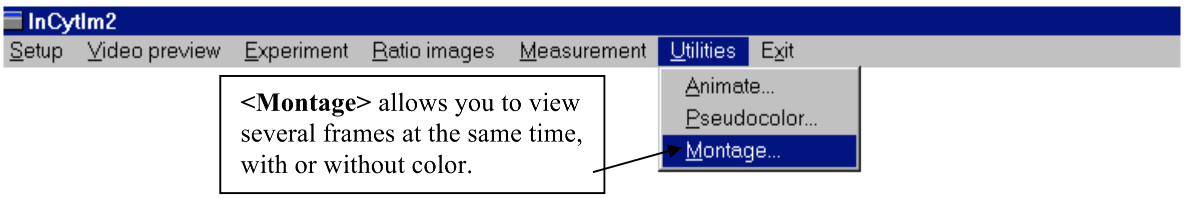
Animate is a good way to see how the changes progressed during the experiment. The animate function will replay the experiment for you frame by frame, or you can manually choose the frames you wish to view. This allows you to compare the different images that were captured during the experiment.



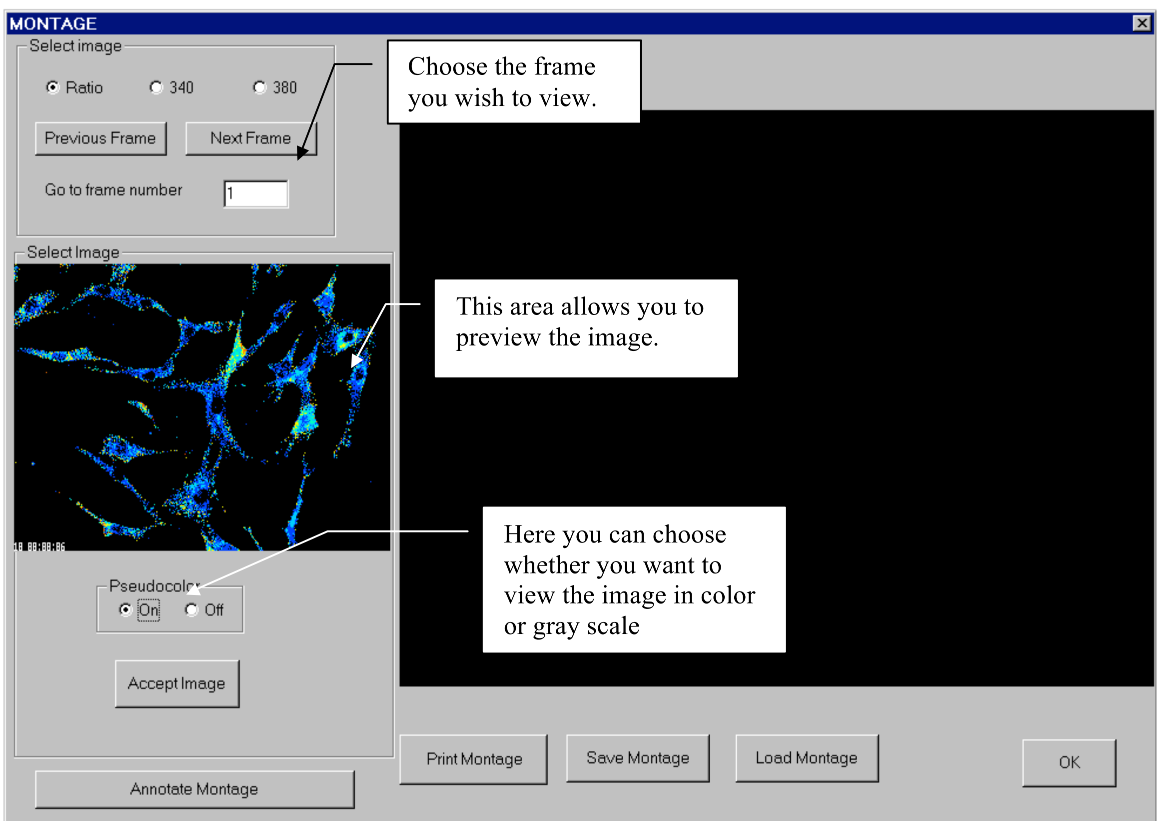
G. UTILITIES

1. Montage

a. Creating a Montage



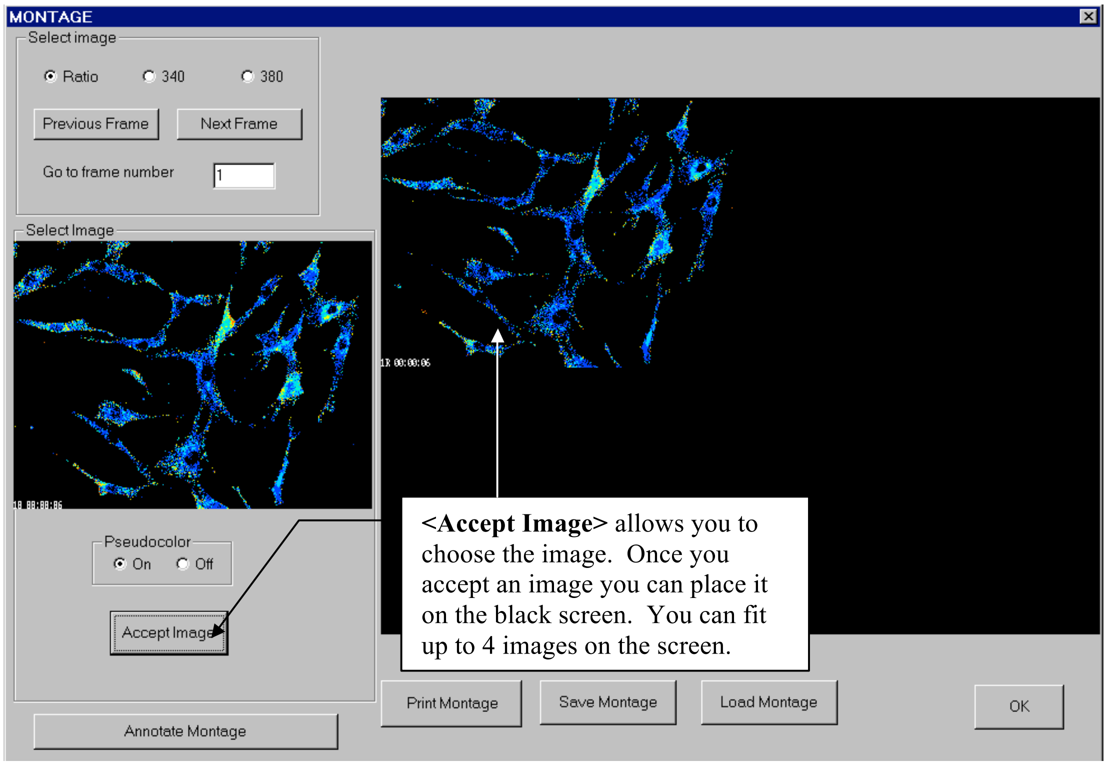
A montage is a good way to display your results. It allows you to display up to four of the images you captured from the experiment. This can help you to demonstrate the changes that occurred during the experiment or present your data in an easy to understand format. These images can be from the ratio, 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.

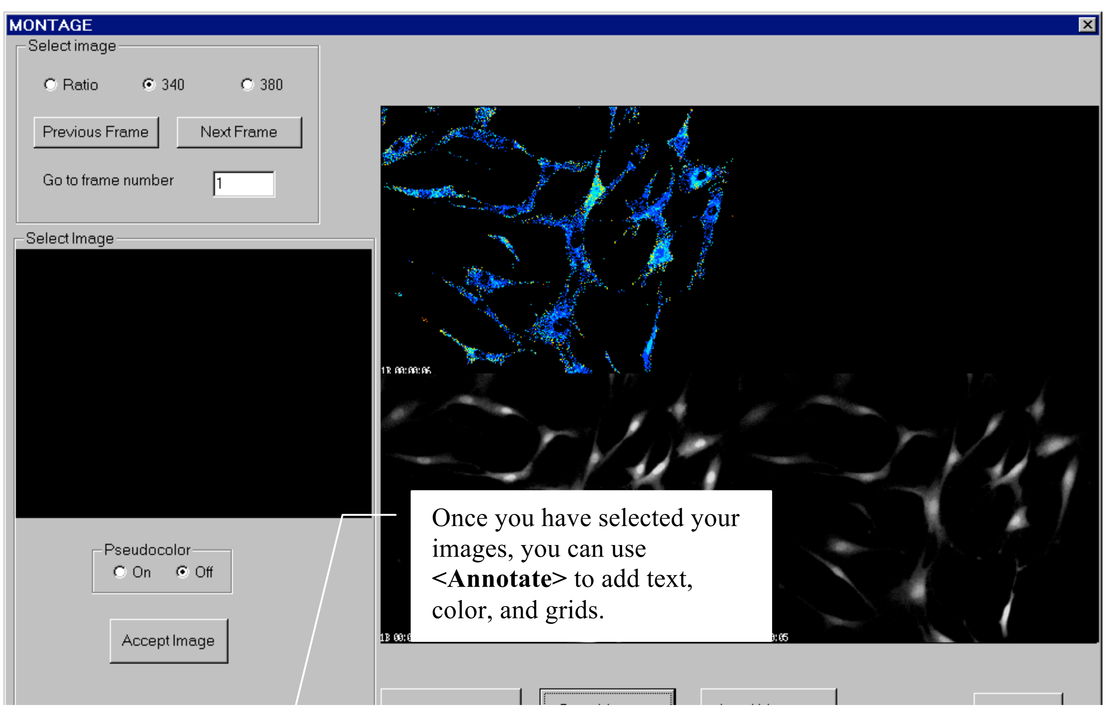


G. UTILITIES

2. Montage

a. Creating a Montage





G. UTILITIES

3. Montage

b. Annotating the Montage