Manual Supplement

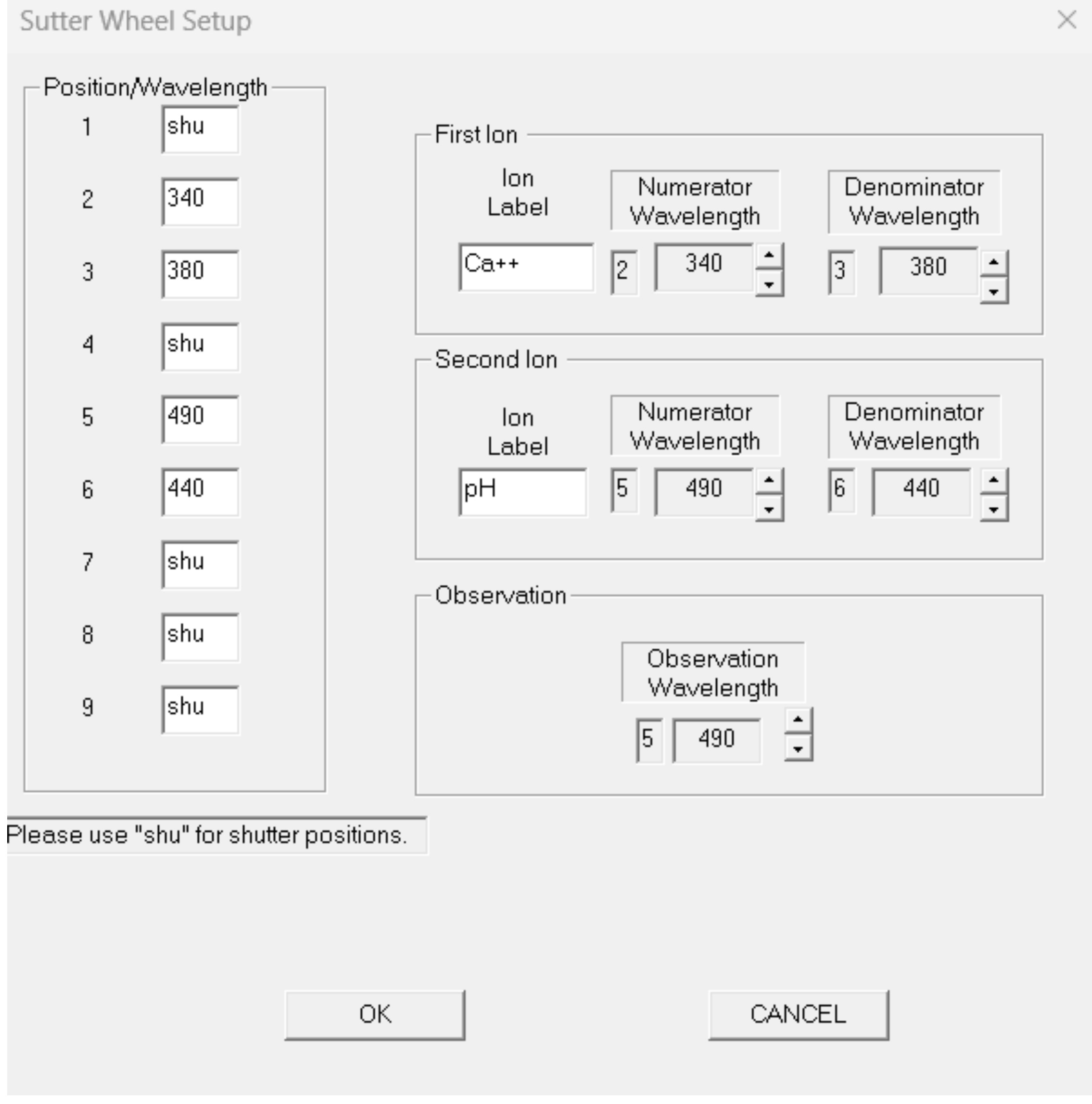
The InCyt Im4™ program operates in a similar manner to InCyt Im2™ program. The difference is that with each step, one is managing two dual-excitation fluorophores rather than just one. This manual supplement will review the key differences in the programs.

Setup

Sutter Wheel Setup

Make sure that the positions for all relevant filters are listed on the left. All blank filter positions should read "shu" for "shutter".

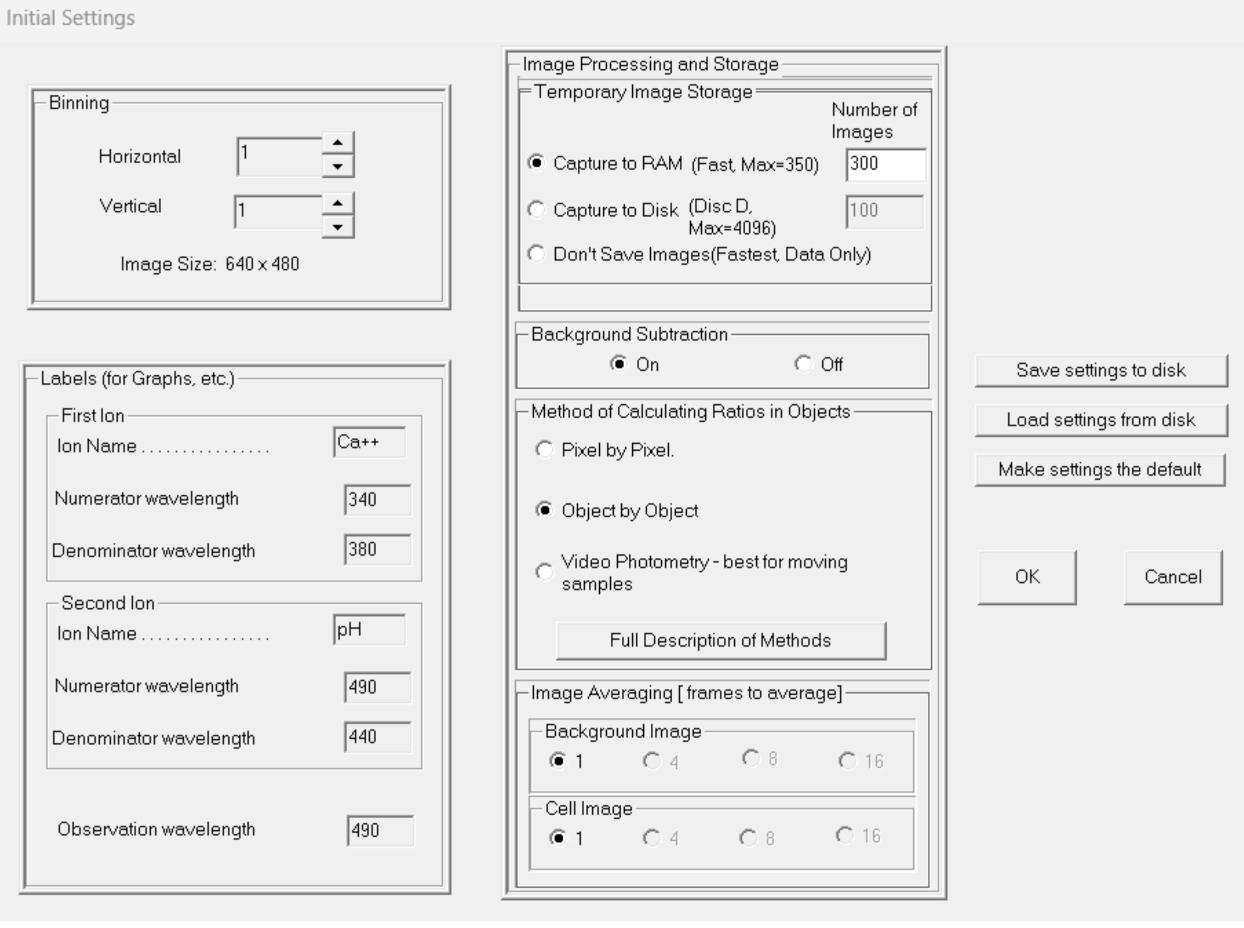
For each fluorophore, enter the ion label (to measure pH, the label should read exactly "pH" in order for the program to display pH levels with decimals). Select both the numerator and denominator filters/wavelength from the list. You may not plan to use an "observation wavelength" (e.g., to identify transfected cells); in this case simply choose any filter on the list for the observation position.



Setup

Initial Settings

You will see your ion labels and wavelengths for both fluorophores flow through from the <Sutter Wheel Setup> to this page. As you would with the InCyt Im2 program, choose whether you want to save images to RAM or Disk (slower, but allows for more images), whether you want to subtract background, and how you want to calculate ratios in your objects. When you are satisfied with your selections, hit <Make settings the default> (or simply <Save settings to disk> if you are only using these settings for a single set of experiments).

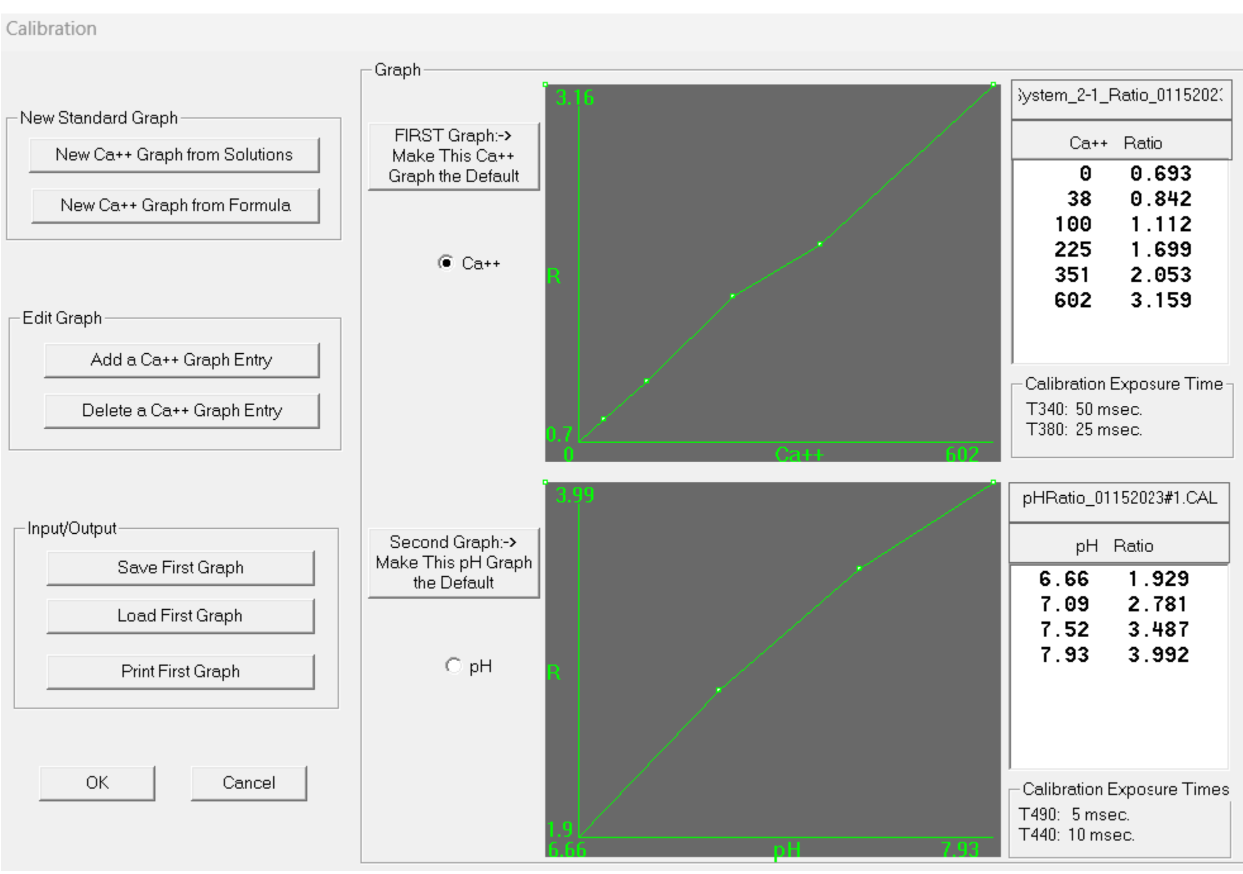


Setup

Calibration

Both calibration curves are shown on the initial Calibration screen. If you already have current calibration curves for your fluorophores, you can use <Load First Graph> and/or <Load Second Graph> from your files. ***Note the radio buttons to the left of the graphs, which will determine which graph you are working with. In this example, as you switch from "Ca++" to "pH", the labels on the buttons along the left will change.***

If you need to create a new calibration curve, select <New xxx Graph from Solutions> and the process is exactly the same as it is in the InCyt Im2 program. Be sure to make each graph the "default" to save yourself time later.

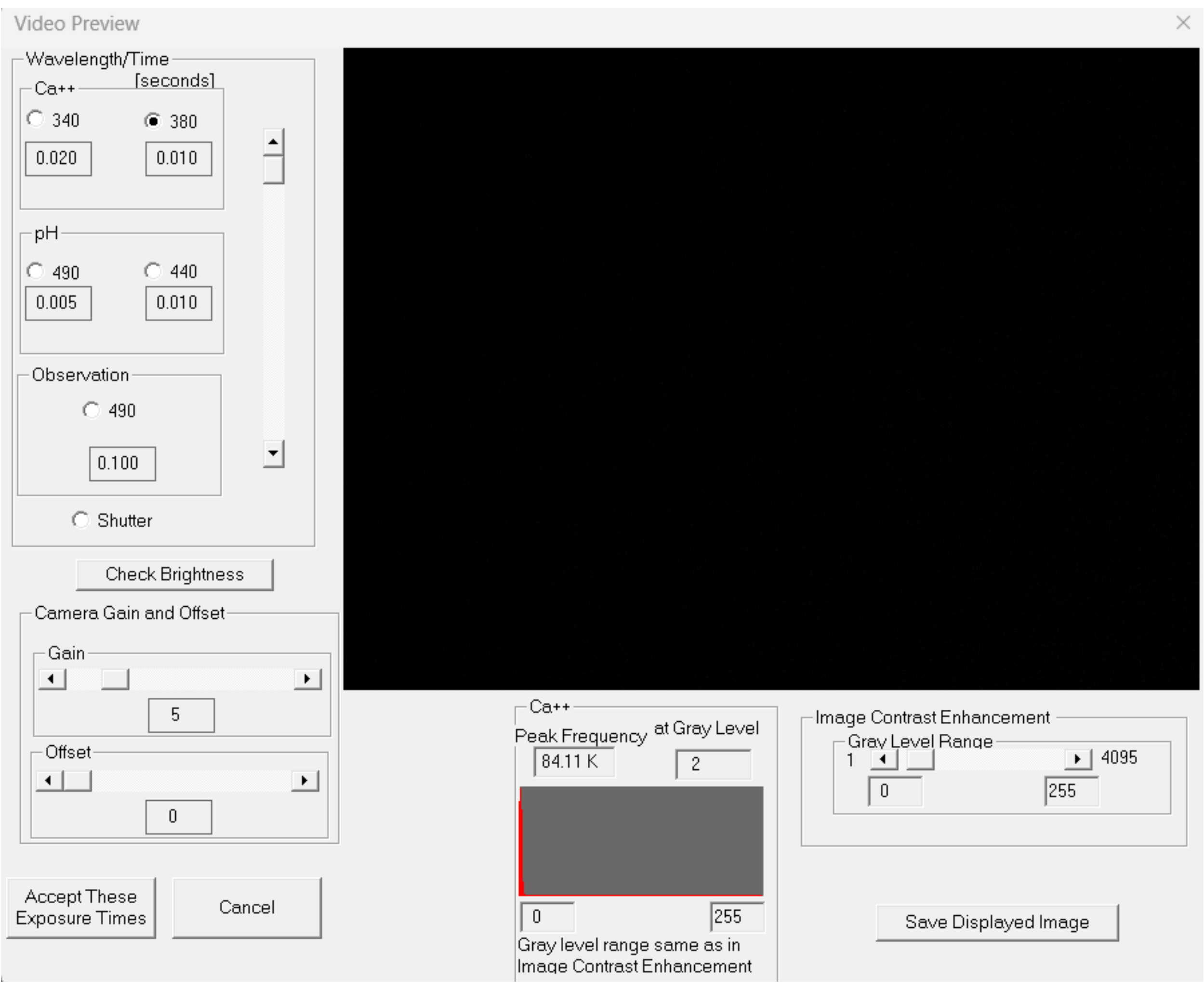


Video Preview

Video Preview works just as it does with InCyt Im2, but for this program, there is just one slider that adjusts the exposure time for the different wavelengths. The slider will change the exposure time for whichever wavelength is selected using the radio buttons.

Remember that your relative numerator/denominator exposure time ratio must match that used in creating the calibration curves…and that ratio may not be the same for each fluorophore/ion. If your exposure time ratios do not match that used in the calibration curves, the program will warn you.

All other settings (e.g., camera gain, image contrast enhancement) apply to all wavelengths.

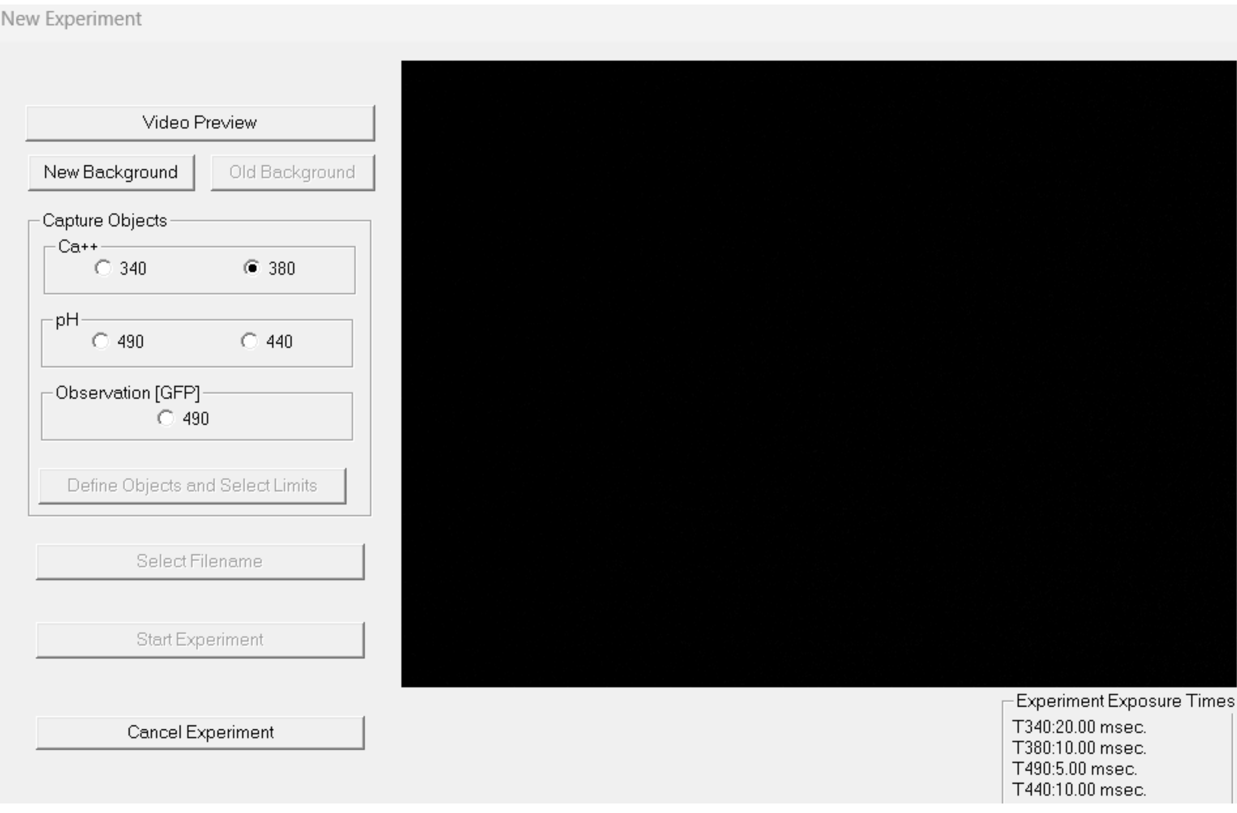


Experiment

New Experiment

Experiments will operate in a similar manner to that in InCyt Im2. For each time point, the system will capture an image for the numerator and denominator wavelengths for each fluorophore. Background images are collected for all four wavelengths and subtracted from their respective images.

After capturing the background, you may define your regions of interest using any of the four wavelengths.

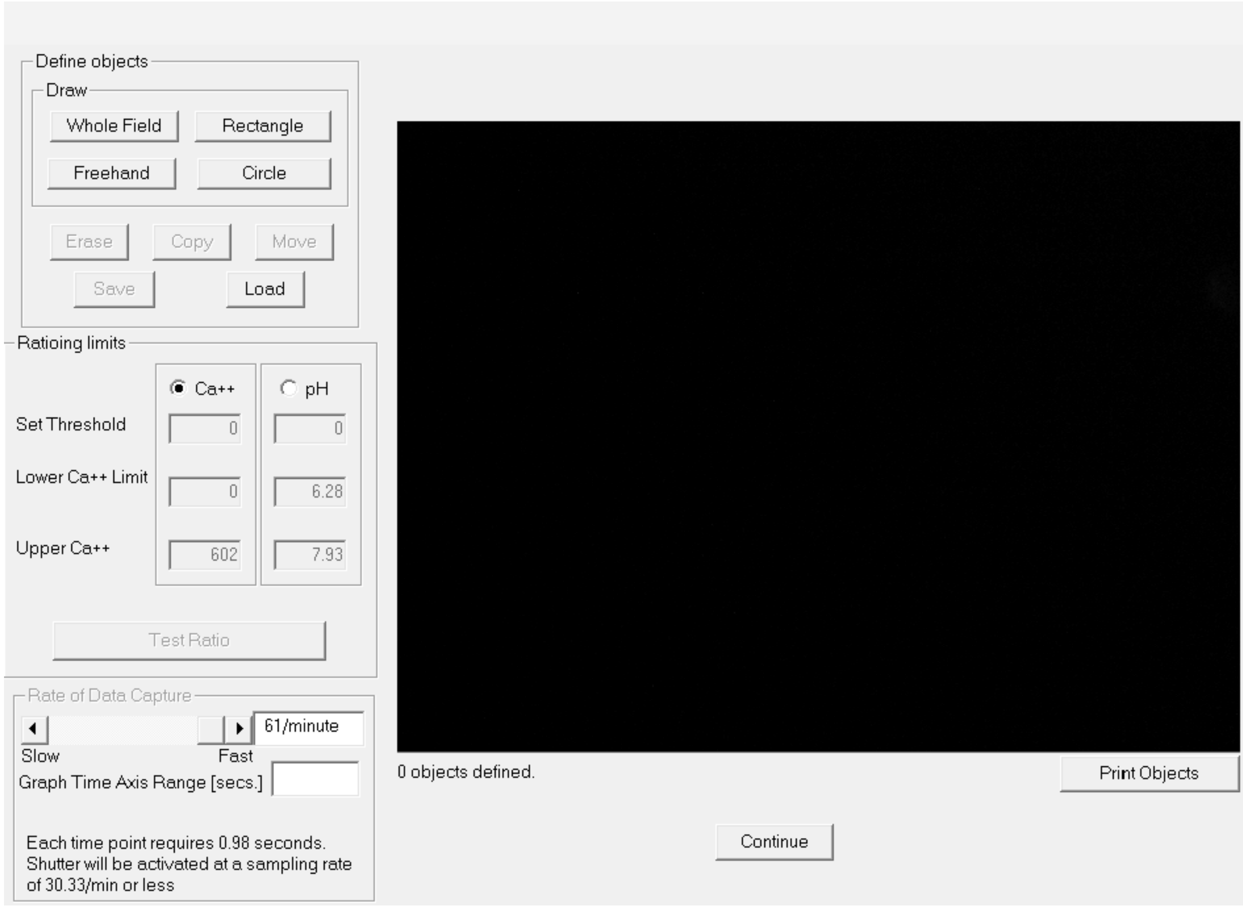


Experiment

New Experiment (define objects and limits)

Selecting objects/regions of interest is the same process as in InCyt Im2. However, you will need to separately select your Threshold, Lower Limit, and Upper Limit for each ion separately. Again, the radio button in the <Ratioing Limits> section of this screen will allow you to set and test each ion separately.

Select your data capture rate and time range for the graph displayed during the experiment as you do in InCyt Im2. Note that because you are now capturing data for two ions, your maximum data capture rate will be slower than it is when measuring a single ion in InCyt Im2.

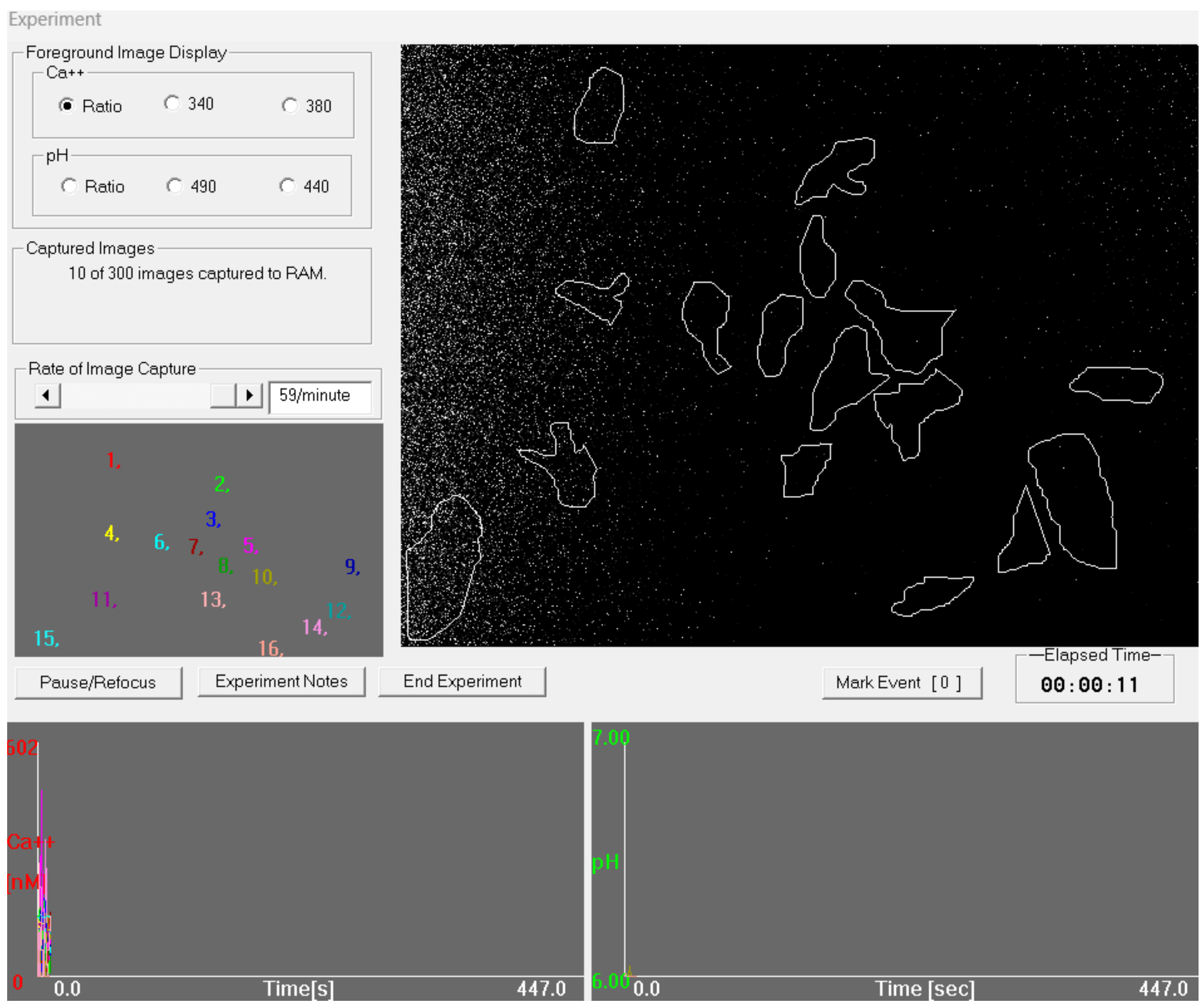


Experiment

During the Experiment

While the experiment is running you have the option of viewing your cells with any wavelength or with either ratio. The level of both ions is graphed at the bottom.

Note that the placement of buttons such as <Mark Event>, <Pause/Refocus>, and <End Experiment> are in different positions than they are with InCyt Im2.

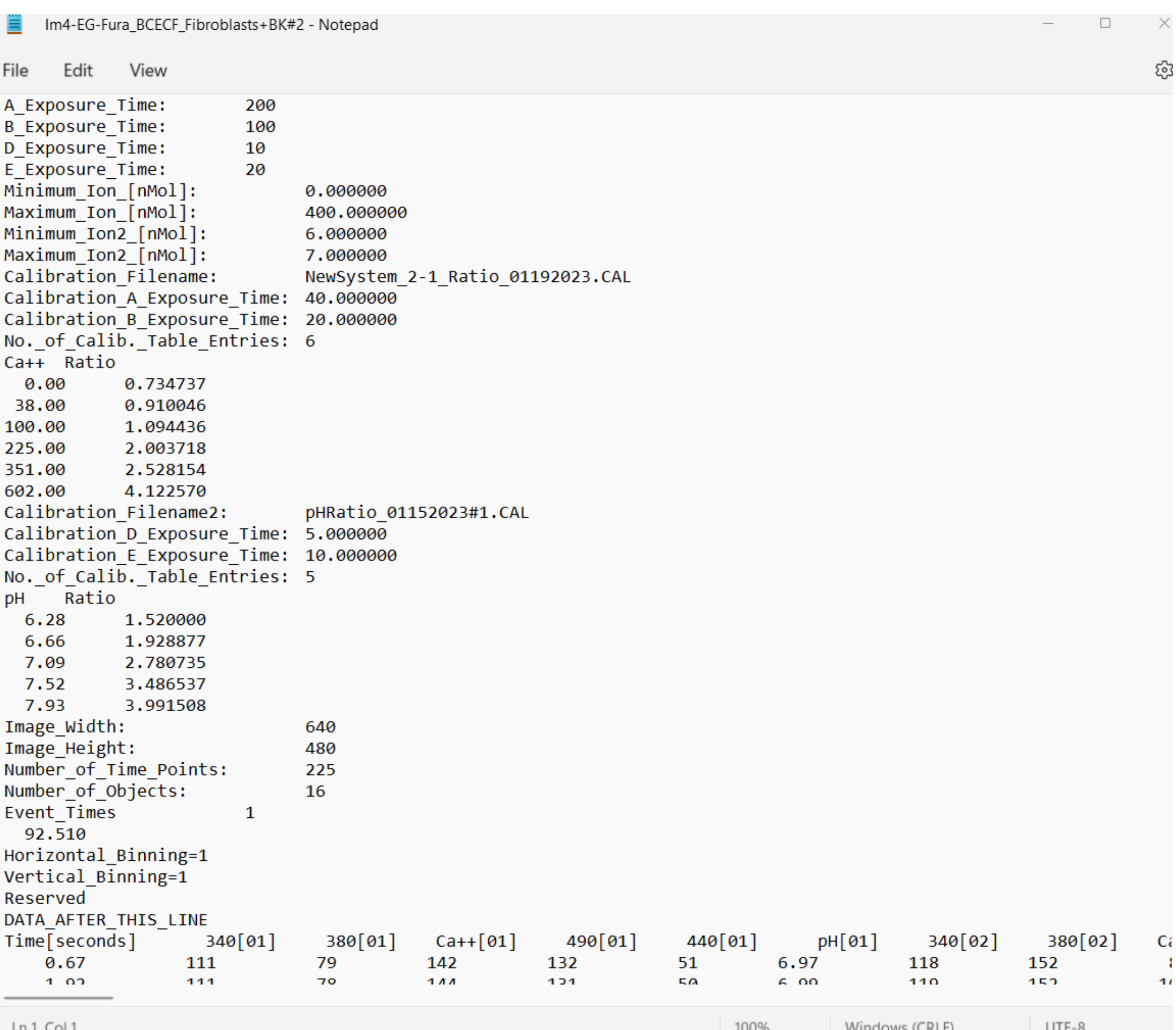


Measurement

View Data

The data file from the experiment contains all the information from the experiment for both ions. The measurement data is laid out as follows:

- Rows: time points
- Column order:
  - Each object
  - Numerator gray level, denominator gray level, and calculated concentration for the first ion
  - Numerator gray level, denominator gray level, and calculated concentration for the second ion



Im4-EG-Fura_BCECF_Fibroblasts+BK#2 - Notepad

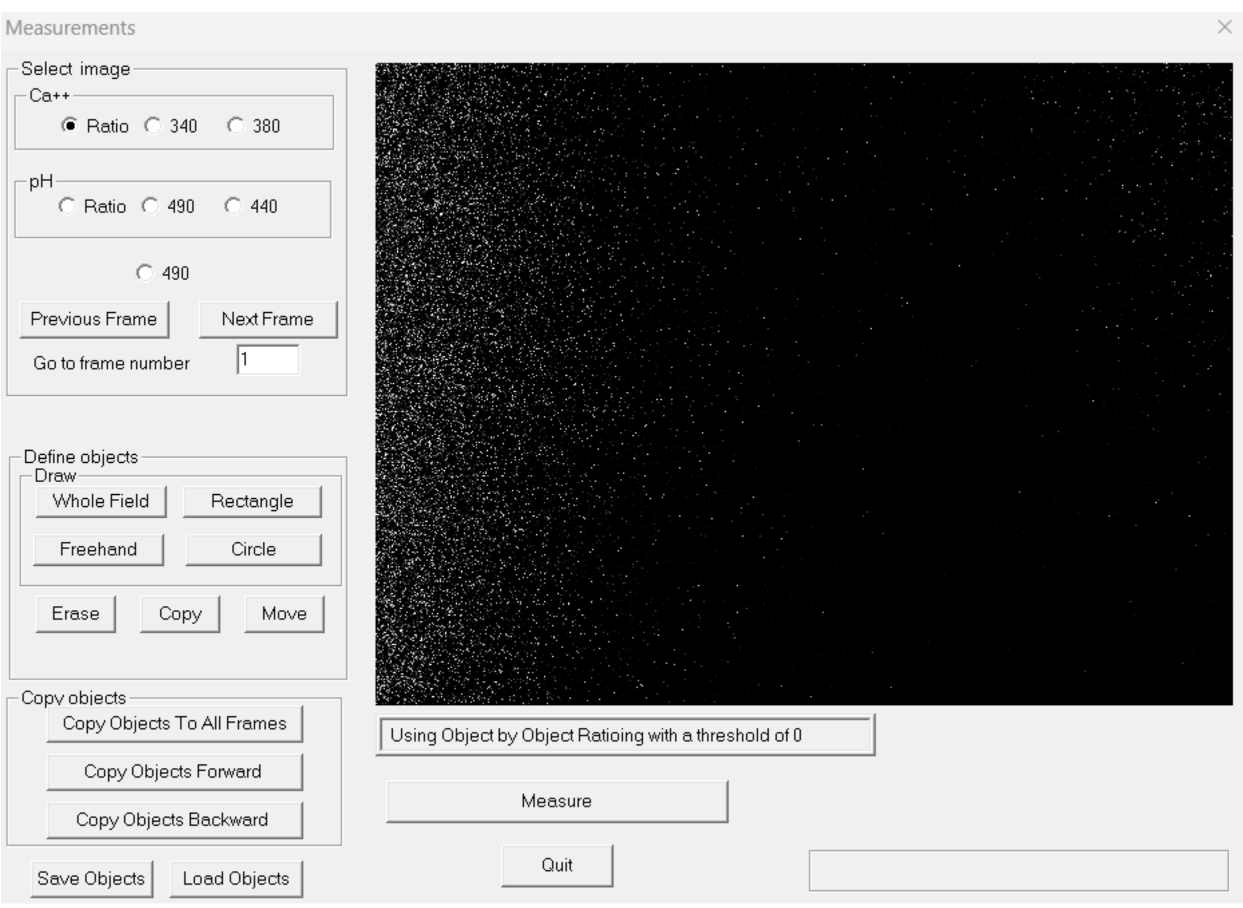
File   Edit   View

```
A_Exposure_Time:        200
B_Exposure_Time:        100
D_Exposure_Time:        10
E_Exposure_Time:        20
Minimum_Ion_[nMol]:           0.000000
Maximum_Ion_[nMol]:           400.000000
Minimum_Ion2_[nMol]:          6.000000
Maximum_Ion2_[nMol]:          7.000000
Calibration_Filename:         NewSystem_2-1_Ratio_01192023.CAL
Calibration_A_Exposure_Time:  40.000000
Calibration_B_Exposure_Time:  20.000000
No._of_Calib._Table_Entries:  6
Ca++  Ratio
  0.00      0.734737
 38.00      0.910046
100.00      1.094436
225.00      2.003718
351.00      2.528154
602.00      4.122570
Calibration_Filename2:        pHRatio_01152023#1.CAL
Calibration_D_Exposure_Time:  5.000000
Calibration_E_Exposure_Time:  10.000000
No._of_Calib._Table_Entries:  5
pH    Ratio
  6.28      1.520000
  6.66      1.928877
  7.09      2.780735
  7.52      3.486537
  7.93      3.991508
Image_Width:                  640
Image_Height:                 480
Number_of_Time_Points:        225
Number_of_Objects:            16
Event_Times             1
  92.510
Horizontal_Binning=1
Vertical_Binning=1
Reserved
DATA_AFTER_THIS_LINE
Time[seconds]      340[01]     380[01]    Ca++[01]     490[01]     440[01]      pH[01]     340[02]     380[02]     Ca
    0.67           111         79         142          132         51         6.97         118         152
```

Measurement

Measure

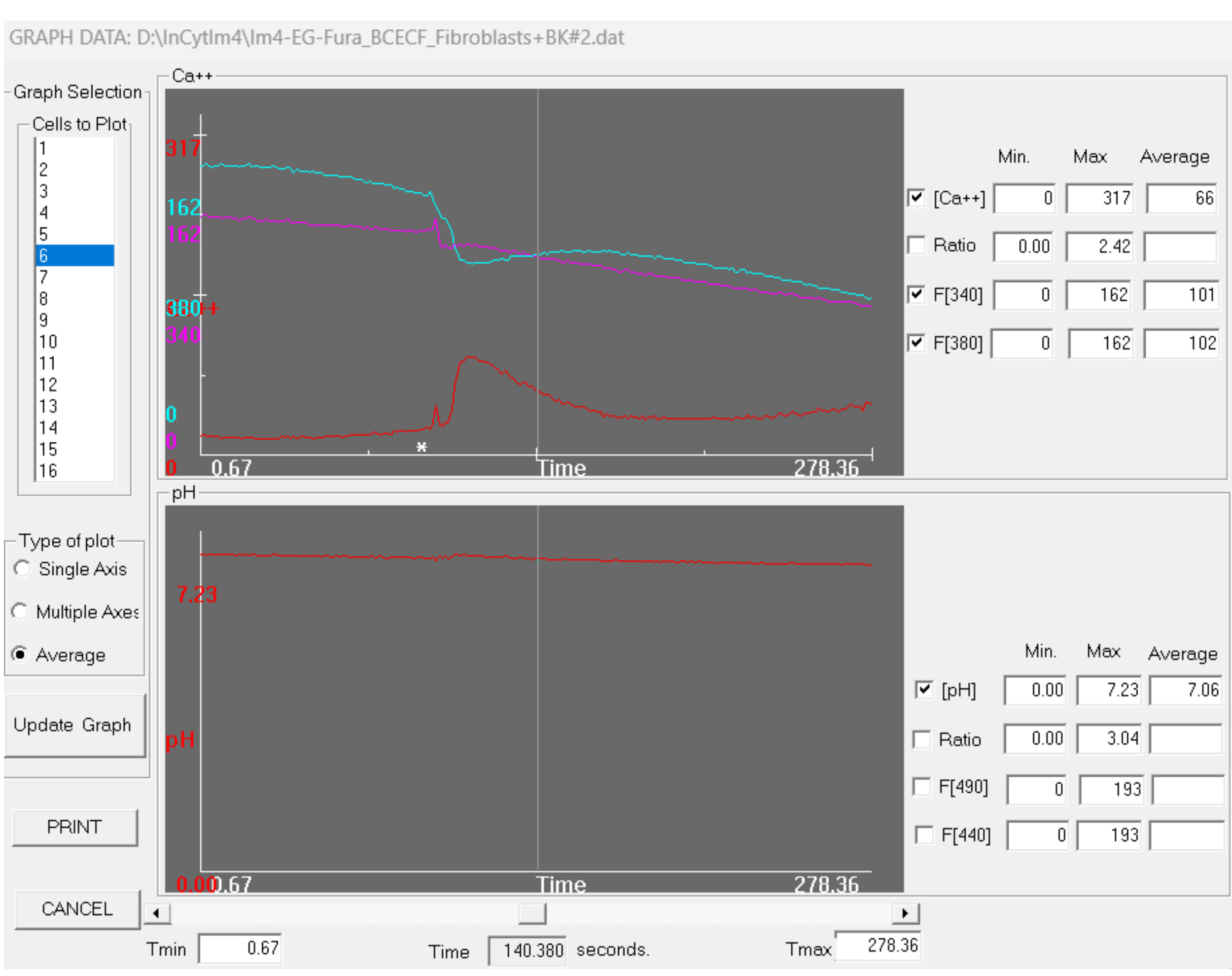
The Measure utility will be familiar. One can view any of the images collected, adjust the objects for any cells which may have moved during the experiment, and measure or remeasure any cell.



Measurement

Graph Data

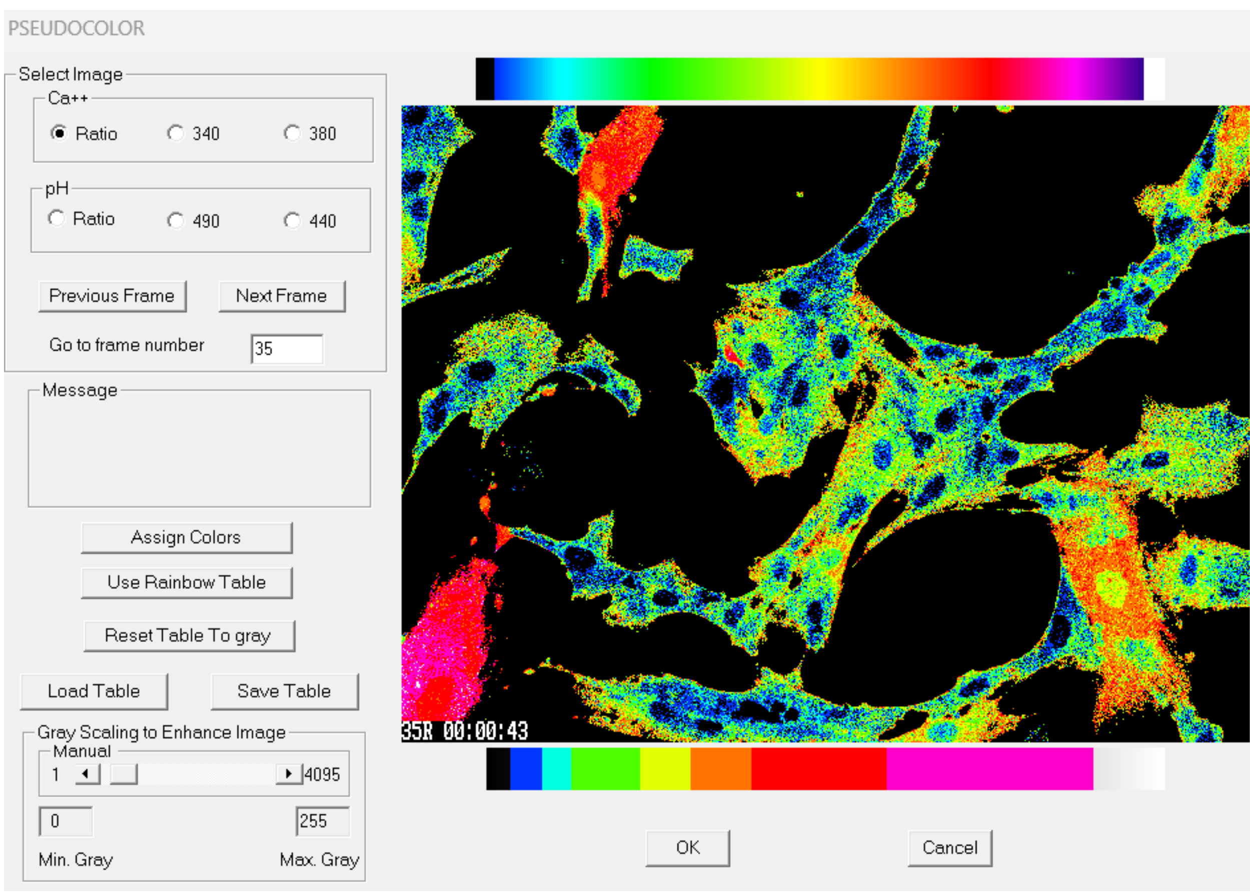
Data for the two ions are graphed separately. The cell(s) and the type of plot chosen apply to both graphs.



Utilities

Pseudocolor

In <Pseudocolor>, one can work with either ion to optimize the color images. However, the program can only work with one color table at a time. If different tables are needed for each ion, they should be created and saved separately.

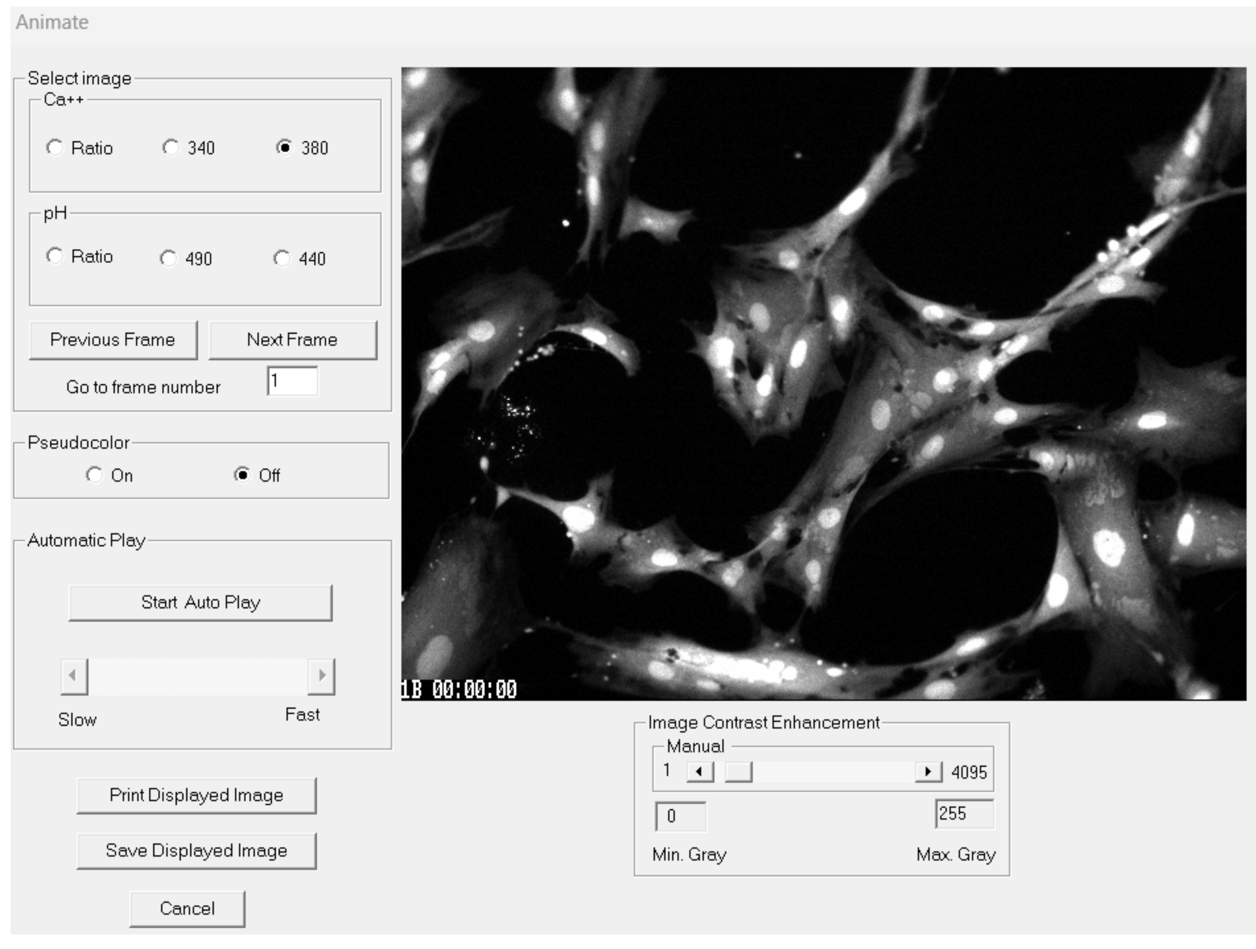


Utilities

Animate

In <Animate>, one can view the sequence of images using any of the four wavelengths, either ratio…and with or without pseudocolor. Two notes about this utility:

1. If you have different psuedocolor tables for each ion and you wish to switch between ions in <Animate>, you will first need to go back to <Pseudocolor> and load the appropriate color table.
2. InCyt Im4 only allows one to save individual images. This program does not have the ability to save the entire sequence of images with one command.



Utilities

Montage

In <Montage> one can select any image collected. However, please note that if you created different pseudocolor tables for each ion, only one table only one table is available when creating a single montage (whichever one was last loaded in <Pseudocolor>).

One can annotate the montage with a calibration scale, but only the first ion's calibration is available in this utility (Calcium in the example below).