GE Healthcare
Life Sciences

IN Cell Analyzer 2000

Cell analysis just got easier

Tutorial Guide
Acquisition Software v4.5

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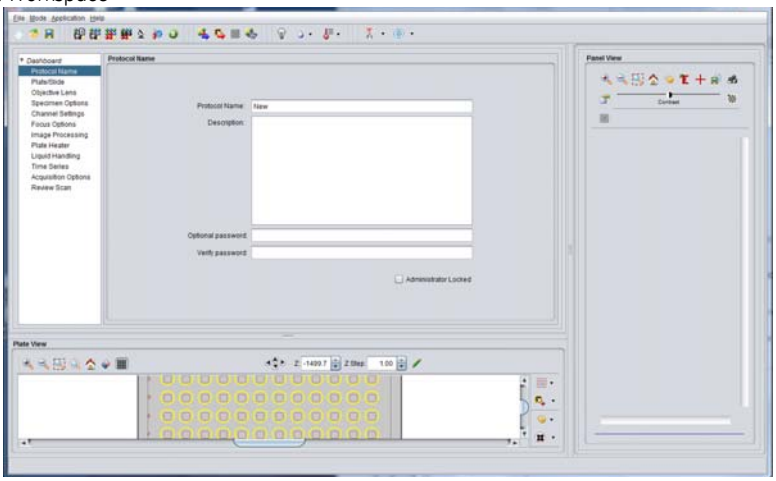
Introduction: General Procedures

The following steps should be followed to start in the creation of acquisition protocols or to start running previously written acquisition protocols.

Turn on the instrument & illuminator  

1. Turn on the workstation and monitor, if necessary.
2. Turn on the instrument by clicking the system power switch. Wait five minutes to allow the *IN Cell Analyzer 2000* to initialize before starting the system software.
3. Start the *IN Cell Analyzer 2000* software by clicking the ***IN Cell Analyzer 2000*** desktop icon.
4. Turn on the lamp from the toolbar ***Illuminator*** icon. The lamp will take 3 minutes to reach optimum efficiency and will turn off after 30 minutes if no imaging has occurred.
5. The acquisition software opens displaying ***Protocol Designer*** wizard, ***Plate View*** and ***Panel View***.

Fig 1. Workspace



Load the plate 

Load the plate into the instrument:

1 Open the plate access door, by clicking the green ***Eject*** toolbar icon. Load the plate so that location A1 is in the top left corner.

2 Click within a well in the ***Plate View*** window to close the plate access door.

The following steps should be followed after the creation of acquisition protocols, viewing the resulting data or shutting down the instrument.

Save the protocol

Before running the protocol, it is recommended that it be saved. Go to ***File|Save as***, provide a *<PROTOCOL NAME>* and ensure that the file type***.xaqp*** is selected.

Protocols are typically stored under the location
`C:\ProgramData\GE Healthcare\IN Cell Analyzer 2000\AQP.`

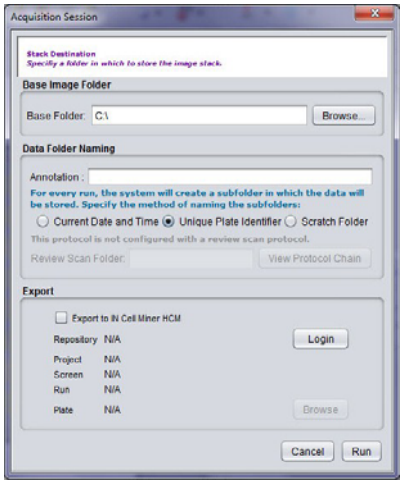
Run the protocol

1 Click the ***Run Protocol*** toolbar icon.

2 Depending on the number of wavelengths defined in your protocol, up to 4 image windows will automatically open. The default display orientation is vertical but this can be changed. If you have defined more than 4 wavelengths in your protocol open ***Acquisition*** mode by clicking the toolbar icon and check the ***Image Layout*** and define orientation and number of windows required and then click ***Run Protocol*** as described above.

3 Define where the images are to be saved and your naming preference.

Fig 2. Acquisition Session



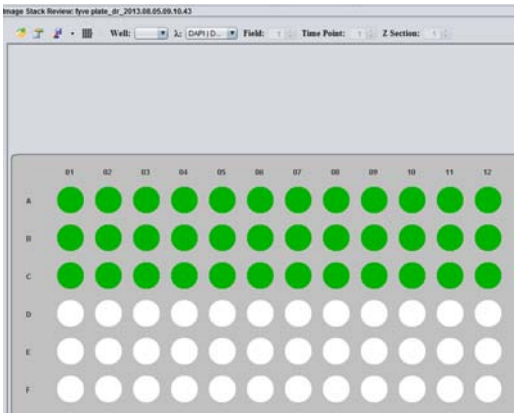
4 To stop a run, click the toolbar ***Stop*** icon.

Data Review mode

Data review mode opens automatically for you to visualize the acquired images. This mode allows individual wells and images to be visualized.

Note: *Data Review mode will not open automatically if the Advanced 2D or Advanced 3D imaging modes are used. If using Advanced 2D/3D you need to wait for processing queue to finish and then open the file in Data Review mode*

Fig 3. Image Stack Review



1 Double-click in well(s) to see the images.

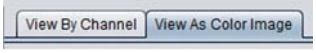
2 View thumbnail images by right clicking the option on the ***Image Stack Review*** window and select thumbnails from the list of options. Selecting Acquisition state will return to original plate view.

3 To define fused images, highlight wells by clicking **CTRL | `left-click`** on the desired wells (a magenta ring will appear around the green well) click the arrow on ***Define Fused Image*** toolbar icon to open the ***Define Wavelength*** window to select wavelength colors.

4 Right-click and select ***Save Fused Images*** from the popup menu. Use **`CTRL`** | **`left-click`** to remove magenta ring from around a well. To view the saved JPEG image navigate to run file destination folder.

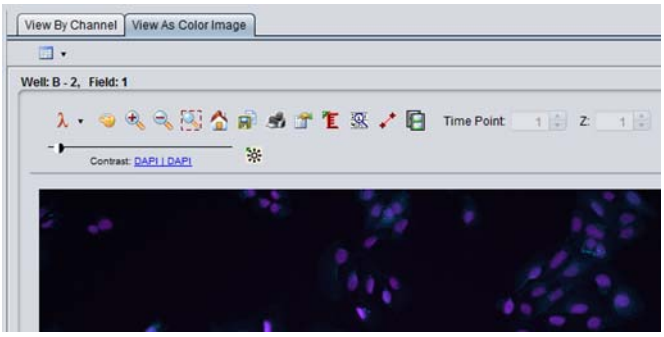
5 Alternatively, to see the individual fused color images, right-click on the well and select ***View as color image*** (up to three can be opened at one time). Or toggle between ***View by channel*** and ***View as color image*** tabs displayed above the image window.

Fig 4.



- Use the ***Wavelength*** icon to select fusion colors and the ***Visuals*** tool to change the contrast of each wavelength. To save the individual fused color images, use the ***Save as*** option.
- When in ***View as color image*** mode additional options such as movies and measures become available.

Fig 5.



Shutting down the instrument

The instrument can be shutdown by either:

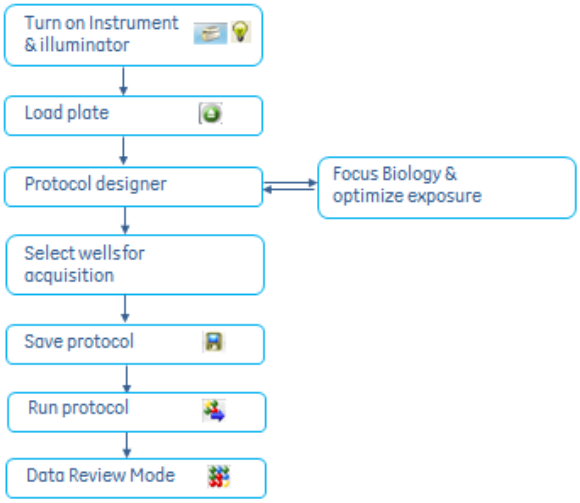
- Select ***Shutdown*** from the ***Applications|Hardware*** menu or
- Close the ***IN Cell Analyzer 2000*** application and depress the power switch to switch the instrument off.

- Turn off the instrument from the main power switch.
- Switch off the workstation and monitor.

1 Standard plate imaging using HWAF

This tutorial describes how to image a typical biology plate (e.g., EGFP-2 × FYVE), containing two fluorescent reporters (e.g., EGFP and Hoechst 33342 Nuclear stain) using the *Hardware Autofocus (HWAF)* feature. A typical workflow is shown in the schematic below.

Fig 1-1. Workflow



Note: *Prior to imaging ensure that the biology plate is equilibrated to room temperature and the imaging surface is clean and scratch free.*

Turn on the instrument & illuminator

- Turn on the instrument as described in *Introduction, Turn on the instrument*.

Load the plate

- Load the sample plate as described in *Introduction, Load the plate*.

Protocol Designer

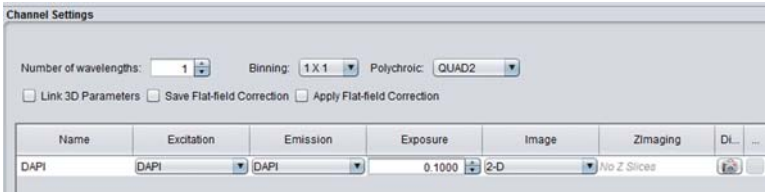
The ***Protocol Designer*** wizard opens at the ***Protocol name*** panel, the default start position for creating a new protocol. Create and optimize an acquisition protocol by navigating the wizard sequentially through each panel. It is not recommended

that the ***Dashboard*** feature be used until new users are more familiar with the software (see tutorial 10).

Fig 1-2. Protocol wizard

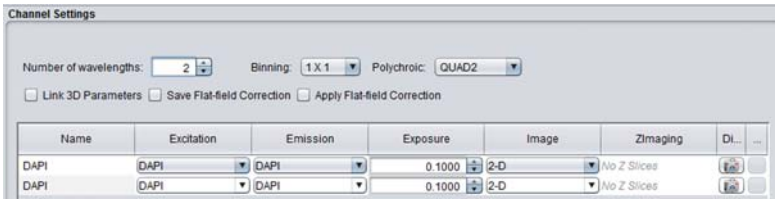
- **Protocol Name** - Protocol name and description of your experiment.
- **Plate/Slide** - Choose a plate from the drop-down menu (e.g.,Greiner µClear). Plate information available here provides the nominal focus position (Z) at the solid/liquid interface.
- **Objective Lens** - Choose an objective from drop-down menu (e.g., 20× objective), with SAC collar setting equivalent to the bottom thickness of the plate (e.g., 170 µm plate thickness = 0.17 SAC setting).
- **Specimen Options** - Available only when you have selected a slide in the **Plate/Slide** card.
- **Channel Settings** - Default page opens with 1 DAPI wavelength selected; 1X1 ***Binning***; QUAD2 ***Polychroic***; 0.1000 ***Exposure***; and 2D ***Imaging*** mode.

Fig 1-3. Channel Settings card - default page



Change number of wavelengths to 2 and a second DAPI wavelength will be added.

Fig 1-4. Channel Settings card - add a second wavelength

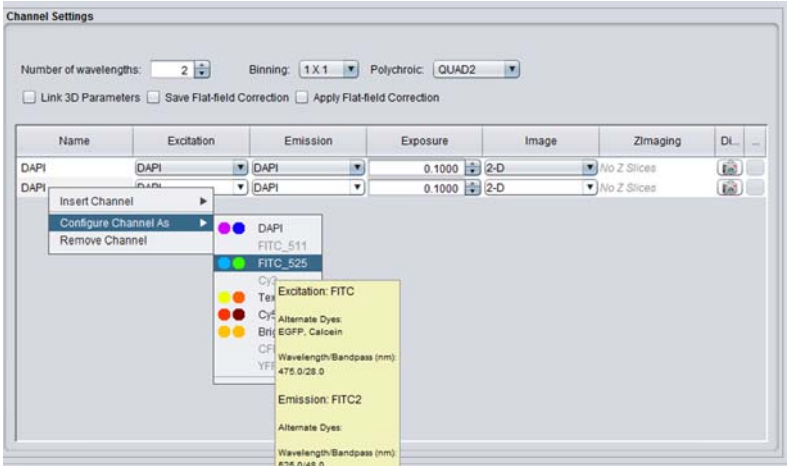


To change the second wavelength from DAPI to FITC click on the name DAPI and choose ***configure channel as*** from the dropdown menu and select FITC_525.

Fig 1-5. Channel Settings card - select FITC

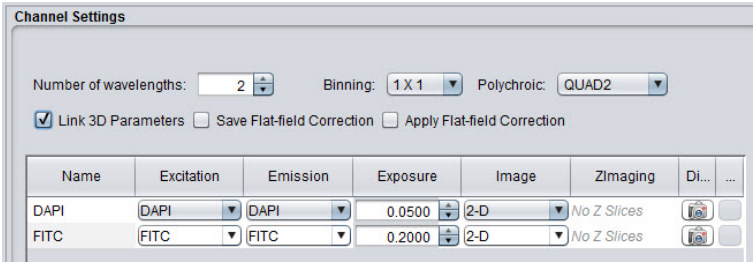
When you hover over FITC_525 information about the Excitation/Emission bandpass and dyes will display as a tooltip.

Fig 1-6. Channel Settings card - viewing the tooltip



Keep *1×1* ***Binning***; *QUAD2* ***Polychroic***; standard *2D* ***Imaging*** mode; and change ***Exposure*** times to the nominal times shown below.

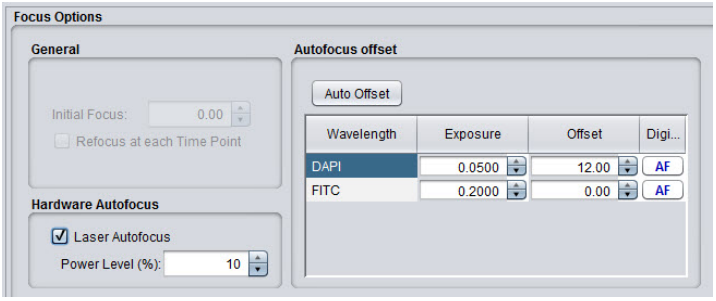
Fig 1-7. Channel Settings card



Note: *You can click on the Excitation and Emission fields to select a wavelength from the dropdown list.*

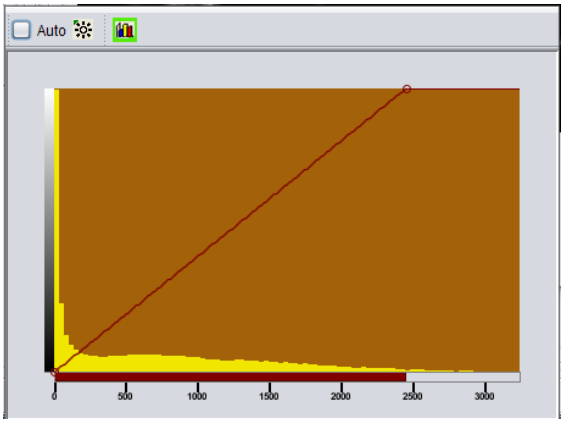
- **Focus Options** - Select ***Laser Autofocus*** (i.e., hardware autofocus), and then optimize the focus and exposure times at each wavelength.

Fig 1-8. Focus card



1. In ***Plate View,*** click within a well where you want to optimize exposure and focus.
2. Click on ***AF*** for *DAPI* wavelength, and visualize the image.
3. In ***Panel View***, open the ***Visuals*** tool by clicking the ***Visuals*** icon.

Fig 1-9. Visuals window



4 Optimize exposure in each channel by adjusting the exposure time, then pressing ***AF*** button to update the image. Assess exposure by looking at the histogram and *MIN*, *MAX*, and *MEAN* signal strength values which are located at the bottom of the panel view.

5 Determine the auto offset by clicking the ***Auto offset*** button. This will provide an offset value for each wavelength.

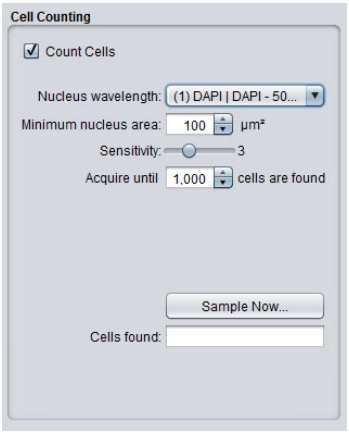
6 Finally, press the ***AF*** button to update the image. Check grey level ranges in each channel to ensure that the images are not over exposed, and are in focus. The offset position can be adjusted manually, if required.

7 Check the exposure & focus settings in a different well (mouse click in a well) and then image at each wavelength by clicking the ***AF*** button in turn to visualize the new image.

- **Image Processing, Plate Heater, Liquid Handling & Time Series options** are not used in this workflow.
- **Acquisition Options** - Select the ***Horizontal Serpentine*** acquisition mode.

  The ***Count Cells*** feature allows a flexible number of fields to be acquired per well until a predetermined number of cells are counted. Mark ***Count cells*** checkbox; define ***Nuclear Wavelength*** (DAPI); ***Minimum Nuclear Area*** (e.g., 100); set ***Sensitivity*** to 3; ***Acquire until*** 1000 cells are found. Press ***Sample Now*** to check that the segmentation parameters are effective.

Fig 1-10. Count Cells panel of the Acquisition card



Ensure a suitable maximum number of fields are defined in the [***Fields to acquire***] panel of the ***Plate View*** window. For example, to count 100 cells counted in 1 FOV, you should select a minimum of 10 FOV to count 1000 cells. Uncheck ***Count Cells*** as it will not be used in this protocol.

- **Review Scan option** are not used in this workflow.

Select wells for acquisition

In ***Plate View***, window, the yellow-colored wells are marked for acquisition. To *deselect wells* use **CTRL** | **SHIFT** | **LEFT CLICK** and drag. To *select wells* use **CTRL** | **LEFT CLICK** and drag.

Save & run the protocol

- Save the protocol as described in *Introduction, Save the protocol*.
- Run the protocol as described in *Introduction, Run the protocol*.
- ***Data Review mode*** will open automatically to review the post acquisition image stacks as described in *Introduction, Data Review mode*.

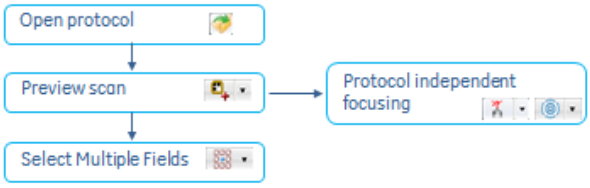
Shutting down the instrument

- Turn off the instrument as described in *Introduction, Shutting down the instrument*.

2 Using preview scan

This tutorial describes the use of ***Preview Scan*** function, scanning one well of a typical biology plate (e.g., plate used in tutorial 1). A typical workflow is shown below;

Fig 2-1. Workflow



Open the protocol

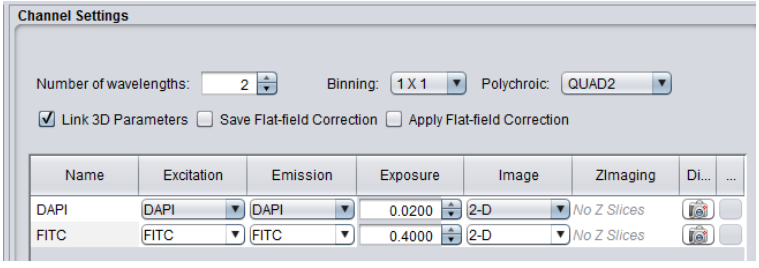
Open the protocol used in Tutorial 1 using the toolbar ***File Open*** command (acquisition protocols are saved under **`C:\ProgramData\GE Healthcare\IN Cell Analyzer 2000\AQP`**).

Run preview scan

1 In the ***Plate View*** window, click within the well that you want to preview.

2 Select the wavelength to use for the preview scan (e.g., DAPI) by clicking the ***Digitize*** button to the right side of the wavelength on the ***Channel Settings card***.

Fig 2-2. Digitize

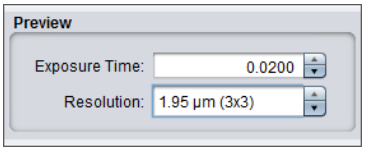


3 Select the objective to use for the preview scan. In this example, the protocol is using the 20× objective, though, a lower magnification can be selected (e.g,. 4×). In general, when performing a preview scan always choose the lowest magnification objective available to allow the preview scan to run quickly.

4 Focus can be achieved in several ways. In this example, focus is set by clicking the ***HWAF*** toolbar icon , and then the ***SWAF*** toolbar icon . Select ***Large*** range (e.g., 200 µm) or focus in the ***Focus Options*** card as in Tutorial 1..

5 Open the ***Setup Preview Scan*** window by clicking the arrow next to the ***Preview Scan*** icon on the ***Plate/Slide View*** window.

Fig 2-3. Set Preview Scan window

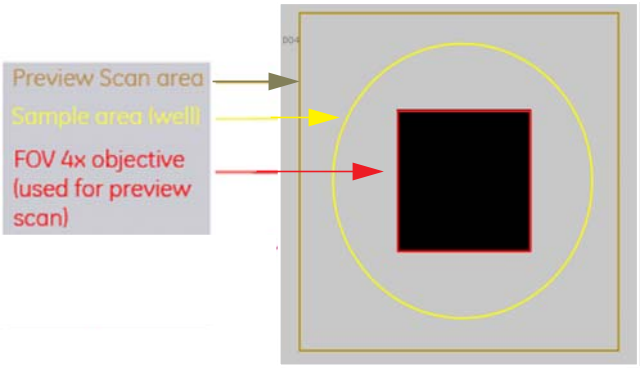


6 Set the ***Exposure Time*** (e.g., 0.020 sec).

7 Set the ***Resolution*** (e.g., 3x3) and then close the window.

8 Click the ***Preview Scan*** icon. Left-click and drag the cursor over the area you want to preview to place a border around the area. If you need to reset the area, click the ***Preview Scan*** icon again.

Fig 2-4. Preview Scan area



9 To run the preview scan, click the ***Preview Scan*** toolbar icon.

10 To stop the scan at any time, click the red ***Stop*** icon on the toolbar.

11 The preview scan images can be viewed in the ***Plate View*** window

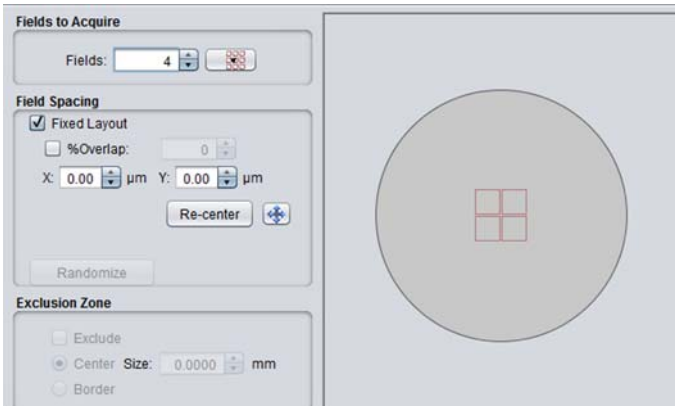
12 It is possible to adjust the contrast display of the preview scan images by choosing the ***Visuals*** icon directly below the ***Preview Scan*** icon on the ***Plate View*** window.

13 Change objective back to the 20X objective.

Select multiple fields for acquisition

- Click the ***Fields to acquire*** icon on the ***Plate View*** window to open the ***Setup Fields to Acquire*** window.
- From the ***Layout*** option, choose four fields from the ***Setup Row/Column*** window. If you are using image stitching, apply a ***10% overlap*** in both ***X*** and ***Y*** axis planes.

Fig 2-5. Setup fields to acquire

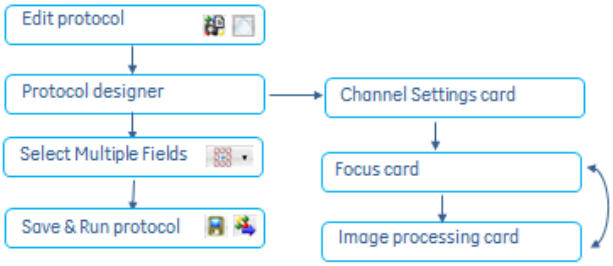


- To move the field overlay over the region of interest (ROI), click the ***Field mover*** icon . The icon displays green allowing one movement per click. Move the fields so that they overlay the ROI located in the ***Plate View*** panel to the left.

3 Using the transmitted light options

This tutorial describes the use of transmitted light options - *Brightfield*, *Phase Contrast* and ***DIC*** -using a typical biology plate (e.g., plate used in Tutorial 1). A typical workflow is shown in the schematic below.

Fig 3-1. Workflow



Since brightfield illumination takes place from the top of the sample, a clear lid or clear plate seal is required. Ensure that the sample has come to room temperature and that the lid/seal is relatively free of condensation.

Edit the protocol

Ensure you are in ***Assay Development mode.*** If not click on the toolbar icon to return to ***Assay Development*** mode. The original protocol is still displayed.

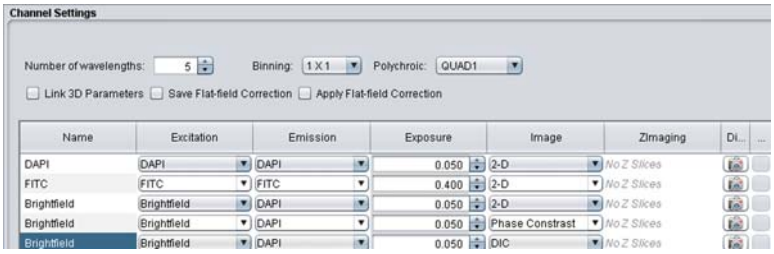
Protocol Designer

Edit and optimize the acquisition protocol by navigating the wizard sequentially through each panel. It is not recommended that the ***Dashboard*** feature be used until new users are more familiar with the software (see tutorial 10).

Fig 3-2. Protocol wizard

- **Protocol Name** - Provide a new protocol name and description for your experiment
- **Channel Settings** - Select 5 wavelengths as described in *Tutorial 1 Protocol Designer -Channel Settings*. Set *1×1* ***Binning***, *QUAD1* ***Polychroic***, add 3 sets of ***Brightfield*** filters, select *2D*, *Phase Contrast* and *DIC* ***Imaging*** modes in turn for the brightfield options and nominal ***Exposure*** times as a starting point as shown in the following image:

Fig 3-3. Channel Settings card



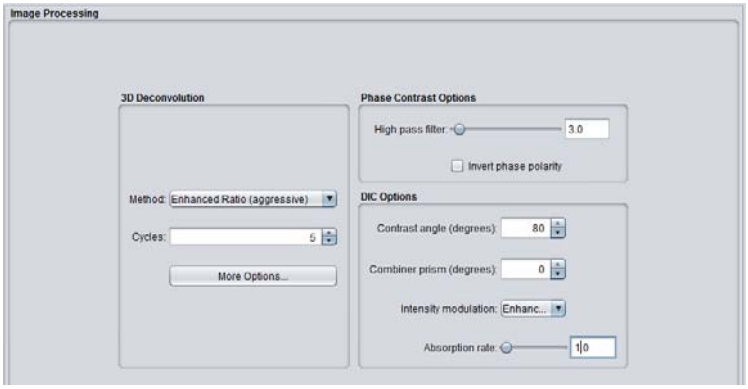
- **Focus Options** - ***Laser Autofocus*** (HWAF) as already selected. Focus the biology, adjust the *Exposure* and set the *Auto-offset* for each channel (as described in Tutorial 1). Ensure that the offset and exposure times for all three *Brightfield* channels are the same to allow a comparison to be made.
- **Image Processing** - Change the settings for Phase Contrast and DIC. In this example the settings are as shown below

Phase Contrast -	High Pass Filter = 3
DIC -	Contrast angle (degrees) = 80
	Combiner Prism = 0

Intensity modulation = Enhanced

Absorption rate = 1

Fig 3-4. Processing card



Toggle between the ***Processing*** and ***Focus*** cards, clicking on AF button next to each wavelength to display the current image until the settings have been optimized.

Note: *Auto offset is not always optimal for brightfield images as it uses a contrast based algorithm so reset offset to 0 and manually adjust*.

Select the field for acquisition

- On the ***Plate View*** click the ***Fields to acquire*** icon.
- Acquire one field at the center of the well by changing the ***Number of fields*** to *1*, press enter and then click the re-center button.
- Select wells for acquisition as described in *Tutorial 1*, *Select Wells for Acquisition*.

Save & run the protocol

- Save the protocol as described in *Introduction, Save the protocol*.
- Run the protocol as described in *Introduction, Run the protocol*, selecting 5 channels in ***Acquisition*** mode prior to selecting run protocol as described in the ***Introduction***.
- ***Data Review mode*** will open automatically to review the post acquisition image stacks as described in *Introduction, Data Review mode*.

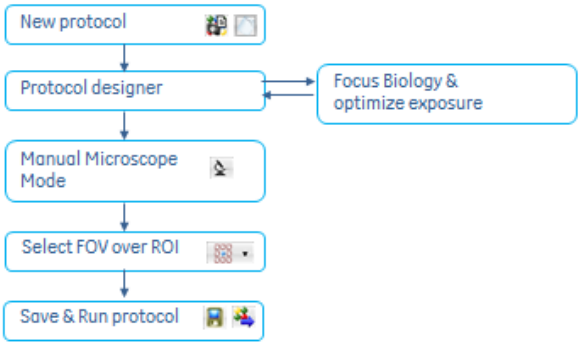
Shutting down the instrument 

- Turn off the instrument as described in *Introduction, Shutting down the instrument*.

4 Investigating image restoration & using manual Microscope mode

This tutorial describes the use of Image Restoration options and Manual Microscope Mode using a typical biology plate (e.g. plate used in tutorial 1). A typical workflow is shown in the schematic below;

Fig 4-1. Workflow



New Protocol

Ensure you are in ***Assay Development*** mode. If not, click the toolbar icon to return to ***Assay Development*** mode and select ***File|New*** or click on the ***Start a new protocol*** icon to create a new protocol.

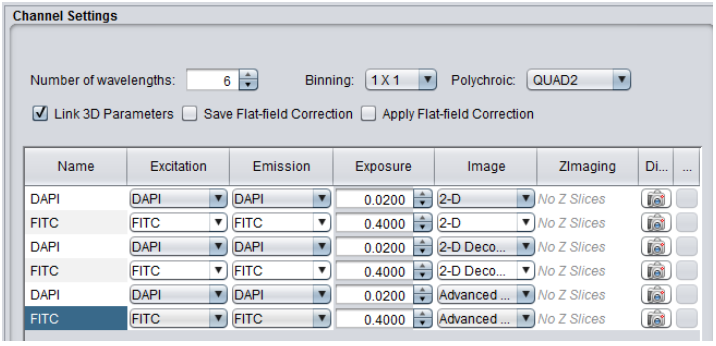
Protocol Designer

The ***Protocol Designer*** wizard opens. Starting at the ***Protocol name*** panel, create and optimize an acquisition protocol by navigating the wizard sequentially through each panel. It is not recommended that the ***Dashboard*** feature be used until new users are more familiar with the software (see tutorial 10).

Fig 4-2. Protocol wizard

- **Protocol Name** - Provide a new protocol name and description for your experiment.
- **Plate/Slide** - Choose a plate from the drop-down menu (e.g.,Greiner µClear). Plate information available here provides the nominal focus position (Z) at the solid/liquid interface.
- **Objective Lens** - Choose an objective (e.g., 40×) and check that the ASAC value is set to 0.17 to match the Greiner µClear plate type.
- **Specimen Options** - Available only when you have selected a slide in the ***Plate/Slide*** card.
- **Channel Settings** - Select 6 wavelengths as described in *Tutorial 1 Protocol Designer -Channel Settings*. Set *1×1* ***Binning***, *QUAD2* ***Polychroic***, add 6 pairs of alternating DAPI and FITC filter sets, ***Imaging*** mode for channels *1&2=2D*, for channels *3&4=2D Decon*, and for channels *5&6=Adv 2D Decon*, and the nominal ***Exposure*** times as shown below as a starting point.

Fig 4-3. Channel Settings card



- **Focus Options** - Select laser autofocus (*HWAF)*. In ***Plate View,*** click in a well to take an image and optimize the ***Exposure*** and ***Offset*** (as described in *Tutorials 1* and *3*) for the DAPI and FITC wavelengths in 2D imaging mode only.

  For comparison, use the same ***Exposure*** time and ***Offset*** for each wavelength for each of the three imaging modes (the effect of 2D Decon can be seen after running the protocol; the effect of advanced 2D can be seen after completion of the processing operation in ***Data Review mode***).

Microscope mode

Microscope mode can be used to find a region of interest before setting up field of view positions.

- Open ***Microscope mode*** using the toolbar icon.
- Hover the mouse along the top edge of the screen to open the imaging menu. Click the pin icon to lock the menu to the desktop.

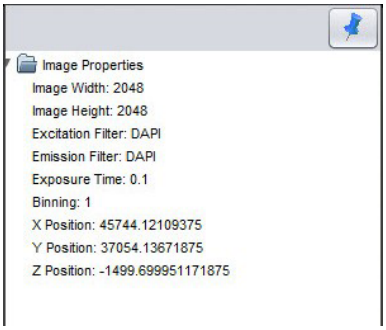
- Press the **<spacebar>** to obtain an image.
- Use the arrow cursor keys to move in the X-Y plane, and the up/down toolbar arrows to move in the Z plane or click on the joystick icon located on the imaging menu to move around the well.

  Note: *To stop using the joystick to move about the well, press* **ESC** *on the keyboard.*

  You can also change the wavelength, exposure time, visual settings, and objective choice by hovering the mouse along the left and right edges of the screen to open the appropriate window.

  To visualize a change, click the **<spacebar>** or the toolbar up/down arrow once.

- Hover along the left edge of the screen to reveal the ***Properties*** window that provides X, Y and Z positioning information.

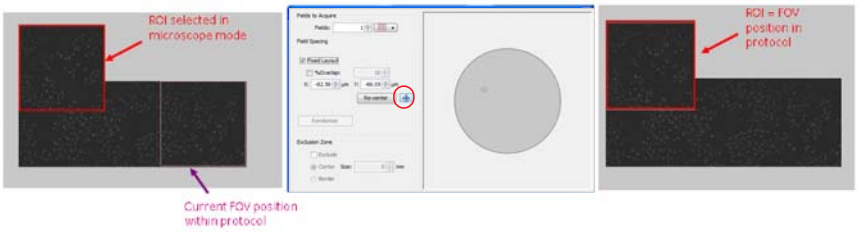


- Hover the mouse along the bottom edge of the screen to open the visualization bar. Clicking the ***X*** icon will exit ***Microscope mode*** and return you to the ***Assay Development mode***.

Move FOV to position over region of interest

- In the ***Plate View***, the solid red outline around a field in a well designates the region of interest (ROI) displayed in ***Microscope mode***.
- To image the ROI, open the ***Set up fields to acquire*** window, and change ***Field*** to *1*.
- To move the field(s), use the ***Field mover*** button (allows one movement per click). Move the FOV to align and overlay the red outline that corresponds to the active imaging area in ***Microscope*** mode.

Fig 4-4. Locating the FOV



- Select the wells for acquisition as described in *Tutorial 1, Select Wells for Acquisition*.
- **Acquisition Options**- Select ***Horizontal Serpenine***

- **Image Processing**, **Plate Heater**, **Time Series**, and **Review Scan** are not used in this protocol.

Save & run the protocol 

- Save the protocol as described in *Introduction, Save the protocol*.
- Run the protocol as described in *Introduction, Run the protocol*, selecting 6 channels using ***Image layout*** in the ***Acquisition Panel View*** if you wish. However images 5 and 6 will be 2D not Advanced 2D images at this point as ***Advanced 2D Deconvolution*** is a post processing operation. The progress of the deconvolution can be seen from ***Applications|Processing Queue***. Upon completion, the images are marked with green ticks and can be viewed in ***Data Review mode*** by selecting ***File|Open*** from the ***Data Review mode*** toolbar and browsing to the saved Run file. Load the Run file into ***Data Review mode*** manually.

  Note: *Data Review mode will not open automatically when the Advanced 2D Deconvolution imaging mode is selected.*

  Note: *Attempting to load the run file before the Advanced 2D Deconvolution have completed presents an error message.*

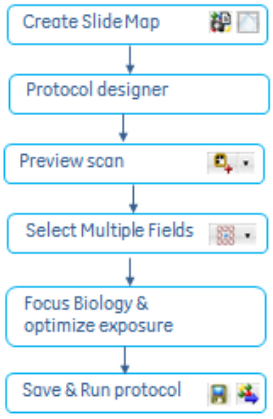
Shutting down the instrument 

- Turn off the instrument as described in *Introduction, Shutting down the instrument*.

5 Slide imaging/location of specimen using preview scan & SWAF

In this tutorial imaging of a tissue section mounted onto a slide is described, first locating the specimen using ***Preview scan*** function and then imaging the specimen at a higher magnification (an example slide could be; FluoCells® prepared slide#4; mouse intestine section (Invitrogen F24631) with Alexa Fluor® 350 WGA, Alexa Fluor 568 phalloidin, SYTOX® Green). A typical workflow is shown in the schematic below.

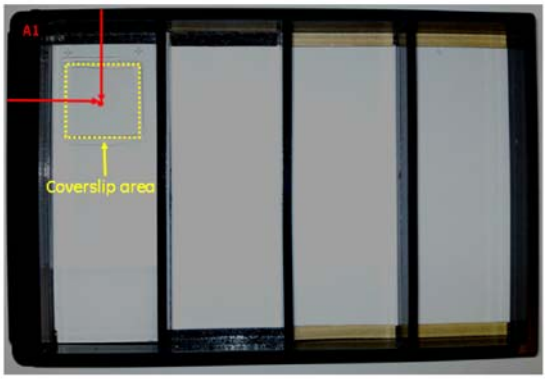
Fig 5-1. Workflow



Create the slide map

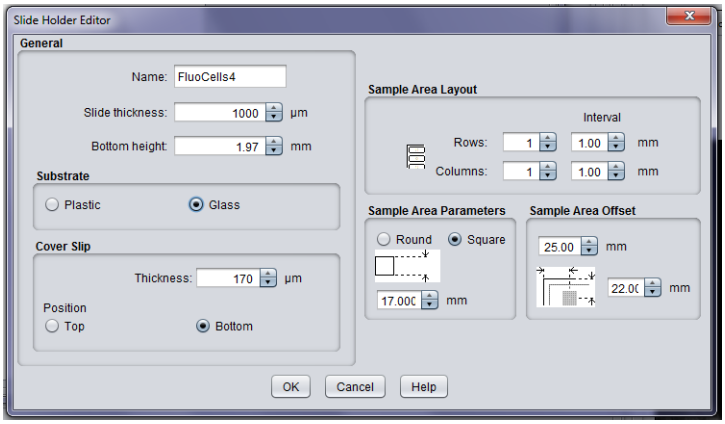
- Place the slide, cover slip down, into the first position of the 4-place slide holder.
- Measure the slide from the edge (horizontal X) and top (vertical Y) to the center of the coverslip (e.g., horizontal = 25mm and vertical = 22mm).
- Measure the coverslip size (e.g., 17mm).

Fig 5-2. 4-place slide holder



- Navigate to ***Application|Plate/Slide Manager*** from the Main menu.
- Locate the ***Slide Holders*** section in the left-hand panel, and click to highlight.
- Click the ***New*** icon on the ***Plate/Slide Manager*** toolbar.
- In the ***Slide Holder Editor*** window, provide the information related to slide and coverslip thicknesses and the measurements that were obtained from the slide in the holder.

Fig 5-3. Slide Holder Editor



- Click ***OK*** to save the slide map and close the editor window.

Load the slide holder

- Click the ***Eject*** toolbar icon to open the plate access door, and load the slide holder with position 1 (containing the slide) facing to the left. Close the access door by clicking in the ***Plate View*** window.

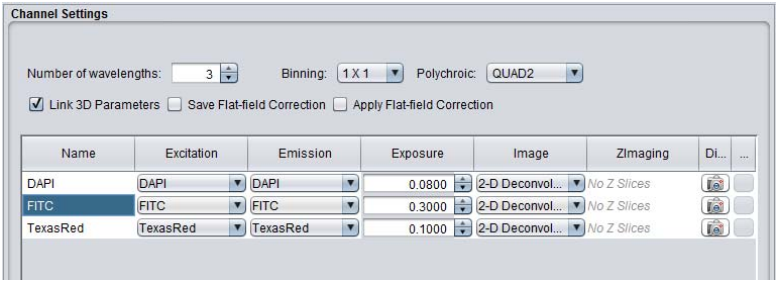
Protocol Designer

Create a new protocol using the ***Protocol Designer*** wizard. Starting at the ***Protocol name*** panel, create and optimize an acquisition protocol by navigating the wizard sequentially through each panel. It is not recommended that the ***Dashboard*** feature be used until new users are more familiar with the software (see tutorial 10).

Fig 5-4. Protocol wizard

- **Protocol Name** - Provide a protocol name and description of your experiment
- **Plate/Slide** - Select the slide option and choose the slide map created above from the list.
- **Objective Lens** - Choose an objective (e.g., 20×) and check the ASAC setting is 0.17 (in this example, a slide with the cover slip on the bottom).
- **Specimen Options** - Enter a value other than 1 µm (e.g., 10 µm). Specify whether the specimen is on the slide or cover slip (cover slip, in this example).
- **Channel Settings** - Using the specimen slide described in the tutorial introduction, the values indicated below provide a good starting point. Add 3 wavelengths as described in *Tutorial 1 Protocol Designer -Channel Settings*.

Fig 5-5. Channel Settings card



Before continuing with the design of the protocol, perform a preview scan using a lower magnification lens to locate the specimen.

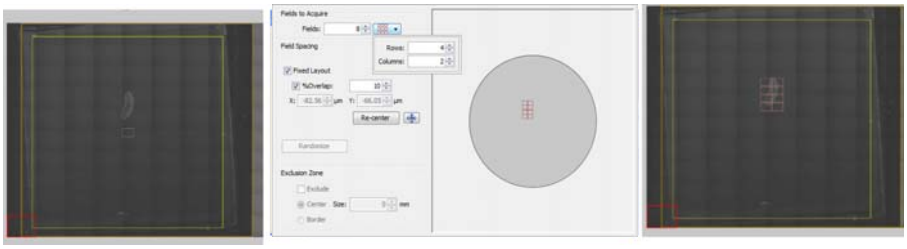
Run a preview scan

- On the ***Plate View*** window, click within the area of the coverslip.
- Select a preview scan wavelength (e.g., FITC [nuclei]) by clicking the ***Digitize*** button at the right-hand side of the FITC row on the ***Channel Settings*** card (see *Fig 5-5* above).
- Select an objective for the preview scan. The protocol is set to use the 20× objective but this can be changed to 4x or10x to perform a faster preview scan.
- Focus can be achieved in several ways. In this example, we use the ***Hardware Autofocus*** (taking you to the air/coverslip interface) or manually enter the nominal focus position (where Z=1500, the skirt height of the slide holder).
- Proceed with the preview scan as described in *Tutorial 2 Run the preview scan*.
- Change objective back to the 20X.

Set up the fields to acquire

- Set up multiple fields for acquisition as described in *Tutorial 2 Select multiple fields for acquisition*. Select enough fields to cover the region of interest (e.g., 8). Adjust the row/column orientation, and apply a 10% overlap in both the X and Y planes if image stitching is required. Move the FOV over the ROI found in the plate view image to the left using the ***Field mover*** button to move the FOV to overlay the ROI (specimen) located in the preview scan.

Fig 5-6. Set up fields to acquire

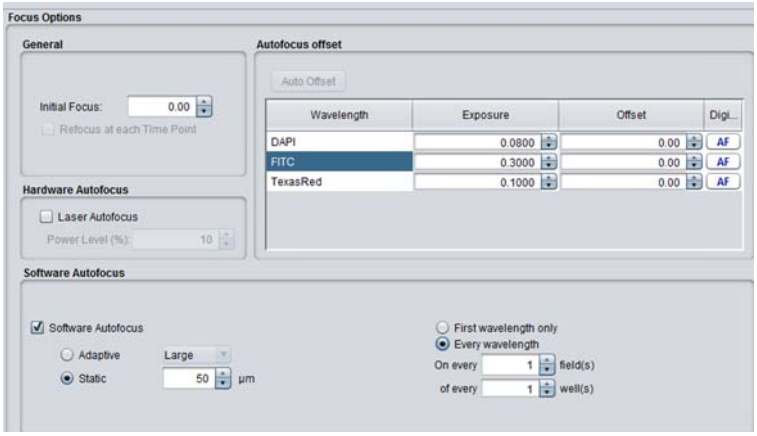


Focus biology & optimize exposure

Continue with the protocol:

- **Focus Options** - Mark the ***SWAF*** checkbox and uncheck the ***HWAF*** box if checked. Use ***Static*** mode = ±*50 µm* at every wavelength.

Fig 5-7. Focus card



- When using SWAF, ensure that the offset value is set to zero for all the channels.
- On the ***Plate View*** window, click on a FOV overlying the visualized specimen. An image in the FITC channel will display in the ***Panel View*** window (If FITC used for preview scan).
- Click the  ***SWAF*** toolbar icon (large range ± 200 µm) or use the Z position up/down arrows on the ***Plate View*** toolbar. Transfer the returned focal position value (Z) to the protocol by pressing the ***Save to protocol***  toolbar icon - this will become the ***Initial Focus*** position used for focusing when the protocol is run.
- On the ***Panel View*** window, open the ***Visuals*** window by clicking the  ***Visuals*** icon to open the tool.
- Optimize the ***Exposure*** in each channel by adjusting the exposure time, and then clicking the ***AF*** button to update the image. Assess the exposure by looking at the ***Visuals*** window and *MIN*, *MAX* and *MEAN* signal strength values located at the bottom of the ***Panel View***.

- **Image Processing, Plate Heater, Liquid Handling & Time Series options** are not used in this workflow.

- **Acquisition Options** - Select ***Horizontal Serpentine***
- **Review Scan** is not used in this workflow

Save & run the protocol 

- Save the protocol as described in *Introduction, Save the protocol*.
- Run the protocol as described in *Introduction, Run the protocol*.
- ***Data Review mode*** will open automatically to review the post acquisition image stacks as described in *Introduction, Data Review mode*.

Shutting down the instrument 

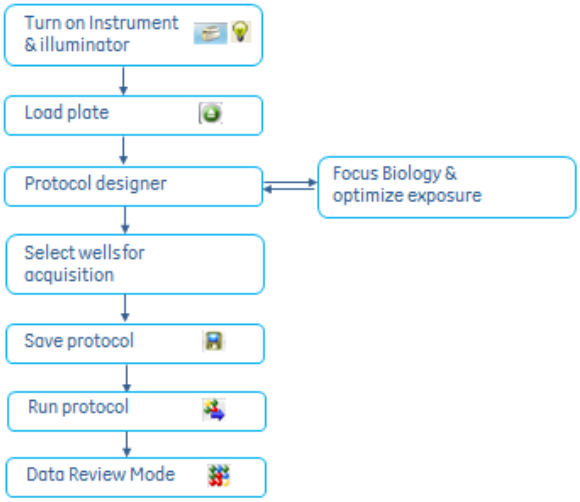
- Turn off the instrument as described in *Introduction, Shutting down the instrument*.

6 Image acquisition using liquid handling/single well time series

This tutorial describes how to use the ***Liquid Handling*** and ***Time Series*** options to acquire a single well time series for a live calcium flux assay using CHO-M1 cells and the agonist Carbachol. A typical workflow is shown in the schematic below

Note: *The timings used in this tutorial are for guidance only and may not be optimal.*

Fig 6-1. Workflow



Prior to imaging ensure that the biology plate is clean and scratch free.

Turn on the instrument & illuminator

- Turn on the instrument as described in *Introduction, Turn on the instrument*.
- Turn on the lamp from the toolbar ***Illuminator*** icon as described in *Introduction, Turn on the instrument*.

Turn on the Liquid Handling module

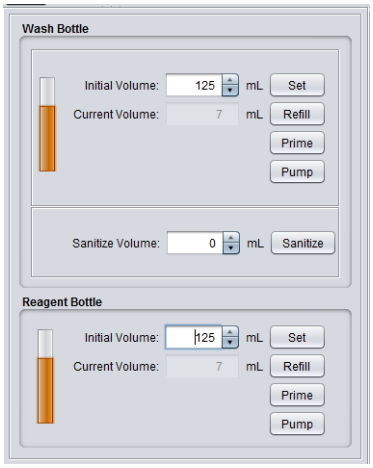
- Click the ***Liquid Handling*** toolbar icon to set the wash and reagent bottle volumes, and prime the liquid handling lines prior to use.

Ensure lines are clean prior to using liquid handling. If in doubt, wash both lines with sterile water using the ***Prime*** function. The lines can then be washed using 50 or 70% ethanol using the ***Prime*** function followed by 2-3 primes with sterile water to ensure all traces of ETOH have been removed.

On completion of your experiment it is advisable to prime both the wash and reagent lines with sterile water to remove all traces of drugs/buffers. The ***Sanitize*** option can be used to flow a volume of sanitizing/wash solution from the wash bottle to the needle. Manually select the volume of cleaning solution to use (1-5ml) and on completion the ***Air dry wash tubing*** option is enabled**.**

- If using the reagent bottle to dispense compound, the reagent line can be primed for use prior to starting the experiment. The prime volume is 5.5ml and this volume needs to be added to the total volume you need for your experiment.
- If there is insufficient fluid volume to complete an operation the system will prevent execution of the Liquid Handling operations.

Fig 6-2. Liquid handling dialog



Load the plate

- Load the sample and compound plates without lids as described in *Introduction, Load the plate*.

Protocol Designer

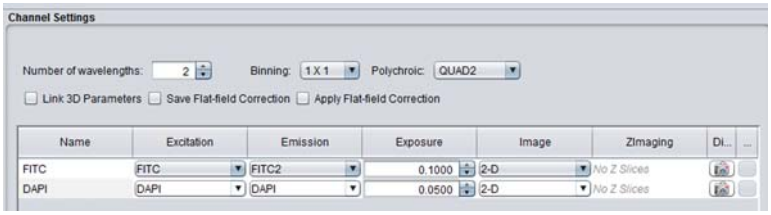
The ***Protocol Designer*** wizard opens at the ***Protocol name*** panel, the default start position for creating a new protocol. Create and optimize an acquisition protocol by navigating the wizard sequentially through each panel. It is not recommended

that the ***Dashboard*** feature be used until new users are more familiar with the software (see tutorial 10).

Fig 6-3. Protocol wizard

- **Protocol Name** - Protocol name and description of your experiment.
- **Plate/Slide** - Choose a plate from the drop-down menu (e.g.,Greiner µClear). Plate information available here provides the nominal focus position (Z) at the solid/liquid interface.
- **Objective Lens** - Choose an objective from drop-down menu (e.g., 10× objective in this example).
- **Specimen Options** - Available only when you have selected a slide in the ***Plate/Slide View*** window.
- **Channel Settings** - Select 2 wavelengths as described in *Tutorial 1 Protocol Designer -Channel Settings*. Set *1×1* ***Binning*** (increasing binning may allow you to acquire images faster); ***Polychroic*** QUAD1 or 2; *FITC* and *DAPI* filter pairs; *2D* ***Imaging*** mode; and ***Exposure*** times as shown below.

Fig 6-4. Channel Settings card

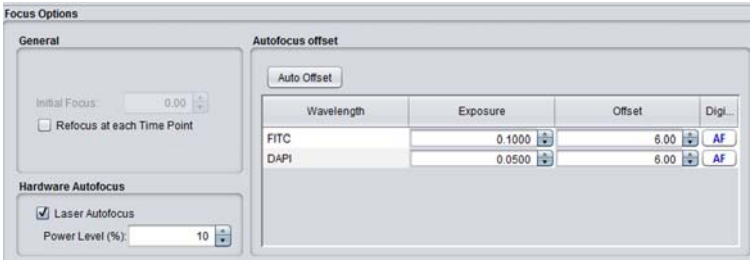


Note: *Both the FITCI and DAPI exposure time can be reduced, but if the FITC exposure time is increased above 0.1 second the 1 second imaging times may not be achieved. The FITC signal will be low at this point. Low*

exposure times in the calcium signaling channel (FITC in this example) will enable you to acquire images faster.

- **Focus Options** - Select ***Laser Autofocus*** (i.e., hardware autofocus), and then optimize the focus and exposure times at each wavelength. Do not select the ***Refocus at each Time point*** for fast kinetic single well assays as it is not required and will add extra time.

Fig 6-5. Focus Options card



1 In ***Plate View,*** click within a well where you want to optimize exposure and focus.

2 Click on ***AF*** for *DAPI* wavelength, and visualize the image.

3 In ***Panel view***, open the ***Visuals*** tool by clicking the ***Visuals*** icon.

4 Optimize exposure in each channel by adjusting the exposure time, then pressing ***AF*** button to update the image. Assess exposure by looking at the histogram and *MIN*, *MAX*, and *MEAN* signal strength values.

5 Determine the auto offset by clicking the ***Auto offset*** button. This will provide an offset value for each wavelength.

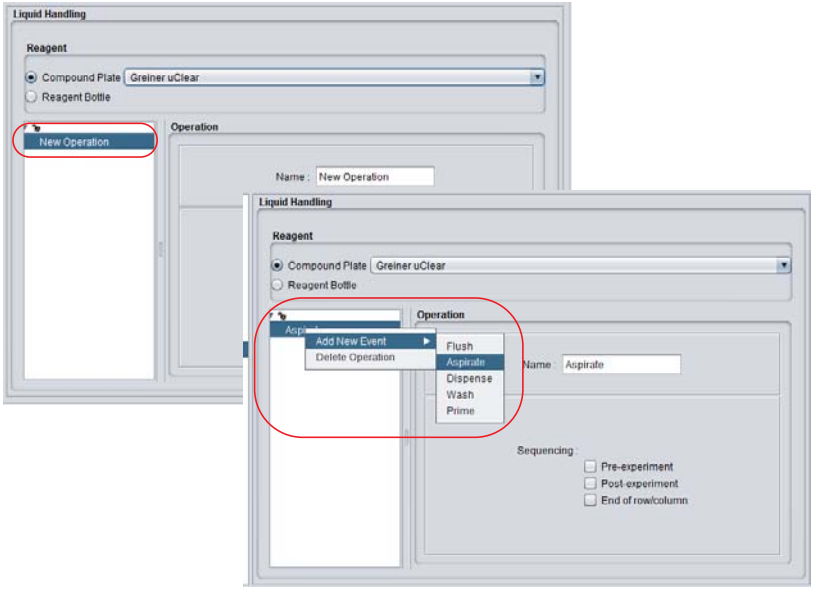
6 Finally, press the ***AF*** button to update the image. Check grey level ranges in each channel to ensure that the images are not over exposed, and are in focus. The offset position can be adjusted manually, if required.

7 Check the exposure & focus settings in a different well (mouse click in a well) and then image at each wavelength by clicking the ***AF*** button in turn to visualize the new image.

- **Image Processing** & **Plate Heater** options are not used in this workflow
- **Liquid Handling -** Mark the ***Compound Plate*** radio button and select a plate type from the drop-down list. Ensure the sample and compound plates are the same size (i.e., both are 96 well plates).

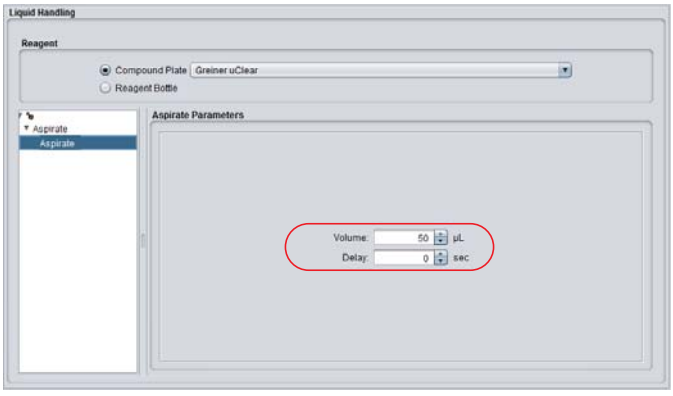
1 In the *Operation* panel, right-click on the root node of the operation tree and select ***Add new operation.*** Name the operation ***Aspirate.*** Right-click on the ***Aspirate*** operation and select the ***Add new event*** option. Select the ***Aspirate*** event from the dropdown menu.

Fig 6-6. Liquid Handling - Aspirate add operation/add event .



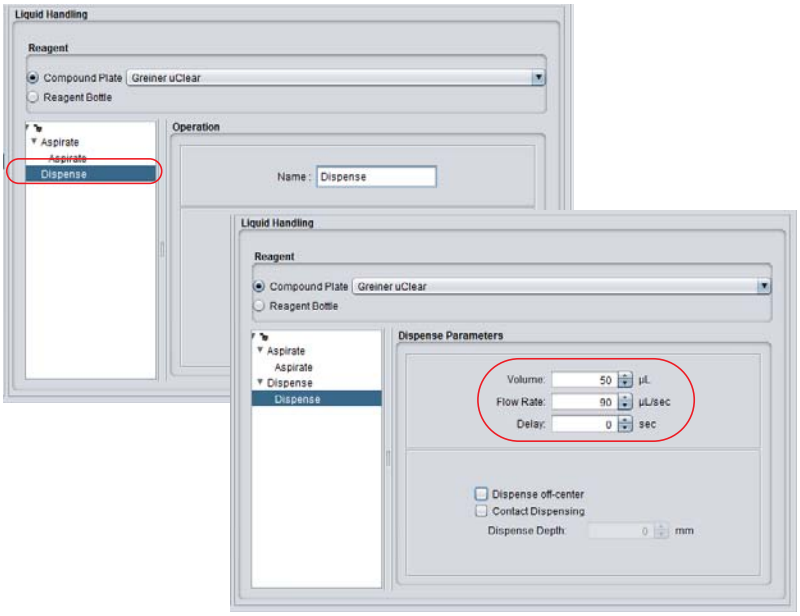
In the *Aspirate Parameters* panel, set the ***Volume*** to *50µl* (volume range: 10-100µl in steps of 10µl), and the ***Delay*** to *0 sec.* (Note that ***Delay*** is normally used for viscous solutions).

Fig 6-7. Liquid Handling - Aspirate add parameters



2 Add a second operation, ***Dispense***, to the Operation tree, by selecting ***Add new operation*** again. Right-click on the ***Dispense*** operation, select the ***Add new event*** option. Select the ***Dispense*** event from the dropdown menu.

Fig 6-8. Liquid Handling - Dispense add event and parameters

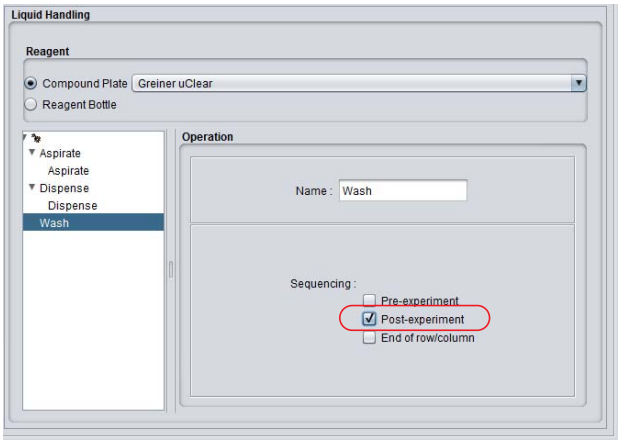


In the *Dispense Parameters* panel enter the values below. Ensure the ***Aspirate*** and ***Dispense*** volumes are equal for this example. (In this protocol we are dispensing into the center of the well using non-contact dispensing.)

Volume	*50µl* (volume range: 10-100µl in steps of 10µl)
Flow Rate	*90µl/sec* (flow rate range: 50-200µl and is cell type dependent)
Delay	*0 sec*
Dispense off-center	*off*
Contact Dispensing	*off*

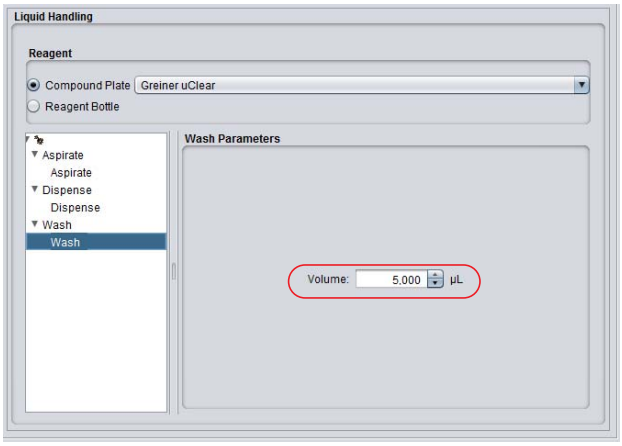
3 Add the third operation, ***Wash***, to the Operation tree, by selecting ***Add new operation*** again. Under *Sequence*, mark ***Post experiment*** as in this example we are adding a dose response curve from compound plate to sample plate going from lowest to highest concentration. This enables faster acquisition because you do not have to wash the needle between wells.

Fig 6-9. Liquid Handling - Wash add operation/post experiment option .



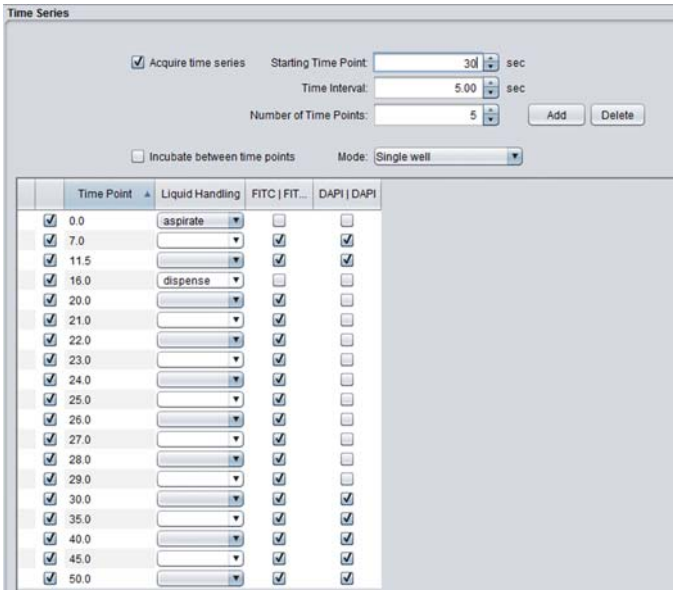
Right-click on the ***Wash*** operation, select the ***Add new event***, and select ***Wash*** from the dropdown menu. In the *Wash Parameters* panel, set the ***Volume*** to *5000µl* (volume range: 1000-5000µl).

Fig 6-10. Liquid Handling - Wash add operation/parameters



- **Time Series** - Mark ***Acquire time series*** and set ***Mode*** to ***Single well.*** Do not mark ***Incubate between time points*** (used with long time courses not fast kinetic imaging)**.**

Fig 6-11. Time series window



- Set the ***Starting Time Point*** to *0 sec*; set ***Time Interval*** to *0.1 sec*; and ***Number of Time Points*** to *1*. Click ***Add***.

  In the table, for Time Point 0.0 select Aspirate from the Liquid Handling drop-down list. Unmark the FITC and DAPI checkboxes so images will not be acquired at this time point. Hover the mouse over Time point 0.0 and the SW will tell you how long it will take to perform this step (~6.66 sec in this case).

- Set the ***Starting Time Point*** to *7 sec*; set ***Time Interval*** to *0.1 sec*; and ***Number of Time Points*** to *1*. Click ***Add***.

- Set the ***Starting Time Point*** to *11.5 sec*; set ***Time Interval*** to *0.1 sec*; and ***Number of Time Points*** to *1*. Click ***Add***.

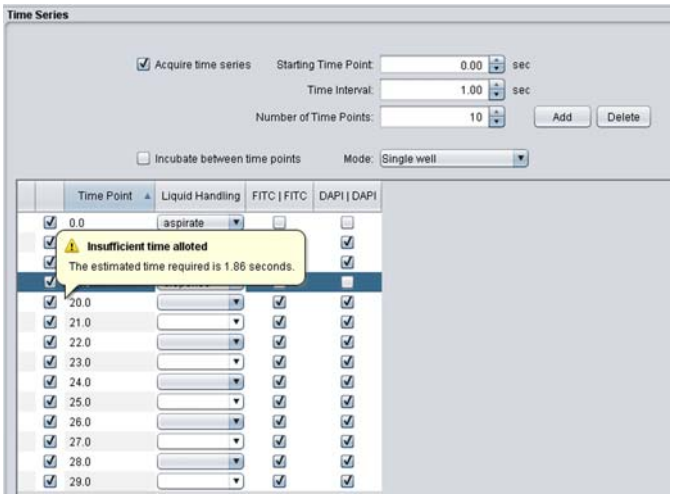
- Set the ***Starting Time Point*** to *16.0 sec*; set ***Time Interval*** to *0.1 sec*; and ***Number of Time Points*** to *1*. Click ***Add***.

  In the table, for Time Point 16.0 select dispense. Unmark the FITC and DAPI checkboxes so images will not be acquired at this time point.

- Set the ***Starting Time Point*** to *20.0 sec*; set ***Time Interval*** to *1sec*; and ***Number of Time Points*** to *10*. Click ***Add***. An error message may be displayed informing you that additional time is required to perform the operation but this clears when the DAPI checkboxes are unmarked.

In the table, unmark the DAPI checkboxes so only FITC images are acquired for the 10 time points.

Fig 6-12.

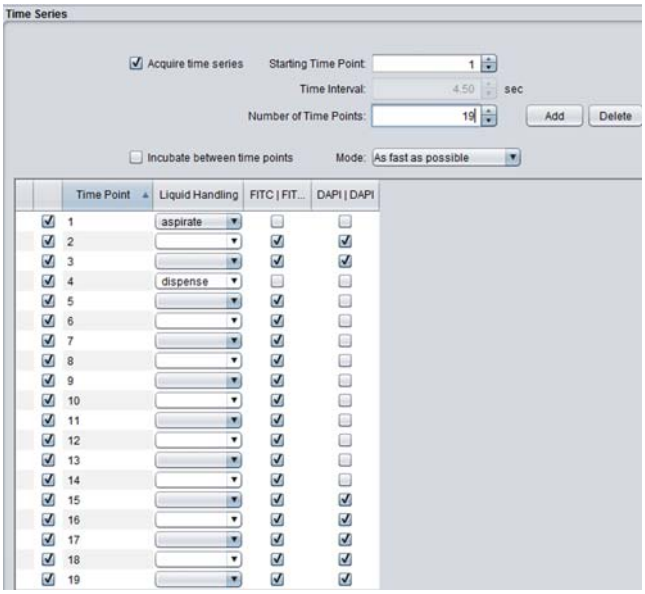


- Set the ***Starting Time Point*** to *30 sec*; set ***Time Interval*** to *5 sec*; and ***Number of Time Points*** to *5*. Click ***Add***.

If you make changes to the protocol after you have defined the time series (e.g., increase/decrease exposure times, change binning) you need to ***Digitize*** an image to update the time series. Failure to do so may results in time points being missed due to timing errors.

As fast as possible mode option:

Fig 6-13. Time series window



Input number of time points required. Select at what time point you require a Liquid Handling operation and select if you require FITC only or FITC and DAPI images. Time interval option is greyed out. By altering the exposure times and binning options in the channel settings page it may be possible to acquire two images/second in this mode.

- **Acquisition Options** - Select the ***Vertical Serpentine*** acquisition mode.

  Count Cells and *Batch Analysis* not used in this example.

- **Review Scan** is not used in this workflow

Select wells for acquisition

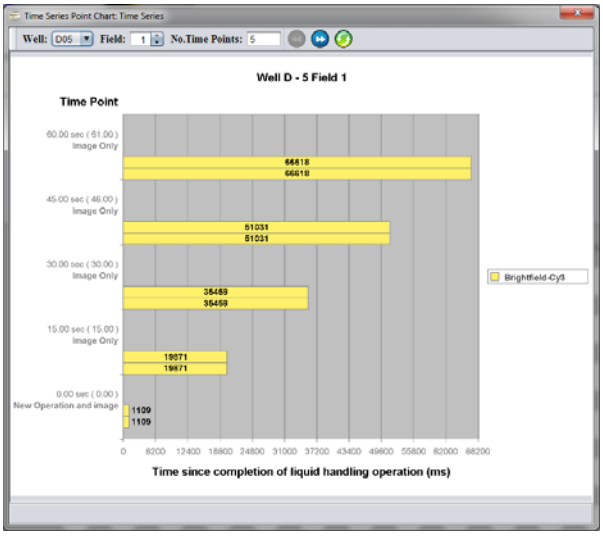
In ***Plate View***, window, the yellow-colored wells are marked for acquisition. To *deselect wells* use **CTRL** | **SHIFT** | **LEFT CLICK** and drag. To *select wells* use **CTRL** | **LEFT CLICK** and drag.

Save & run the protocol

- Save the protocol as described in *Introduction, Save the protocol*.
- Run the protocol as described in *Introduction, Run the protocol*.
- ***Data Review mode*** will open automatically to review the post acquisition image stacks as described in *Introduction, Data Review mode*.

- To view the display time series point chart, right-click on a well to open the popup menu. Select the ***Times series graph*** option to display a graph like the one below.

Fig 6-14. Time series graph



Click on event and the time to complete each step of the event is displayed in elapsed time.

Shutting down the instrument

- Turn off the instrument as described in *Introduction, Shutting down the instrument*.

Appendix: Using the reagent bottle

Using Reagent bottle (example: - when adding agonist to all wells that may have been treated with an antagonist dose response curve).

Turn on the Liquid Handling module

- Turn on the ***Liquid Handling*** module as described at the start of this tutorial.

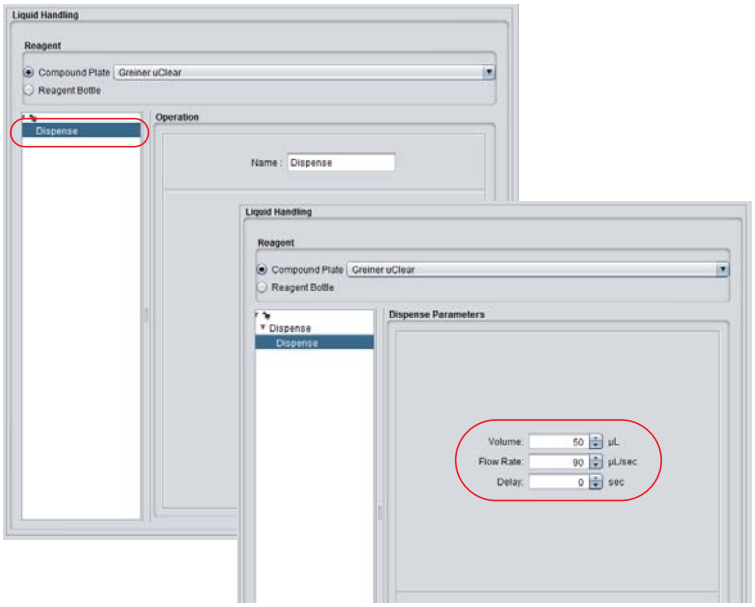
Create Protocol

Create and optimize an acquisition protocol using the ***Protocol Designer***. Navigate through the wizard using the information provided earlier in this chapter up to ***Liquid Handling***.

- **Liquid handling -** Mark the ***Reagent bottle*** radio button.

1 In the *Operation* panel, right-click on the root node of the operation tree and select ***Add new operation.*** Name the operation ***Dispense***. Right-click on the ***Dispense*** operation, select the ***Add new event***. Select the ***Dispense*** event from the dropdown menu. In the *Dispense Parameters* panel, set the ***Volume*** to *50µl*, set the ***Flow Rate*** to *90µl/sec* (for example), and the ***Delay*** to *0 sec*.

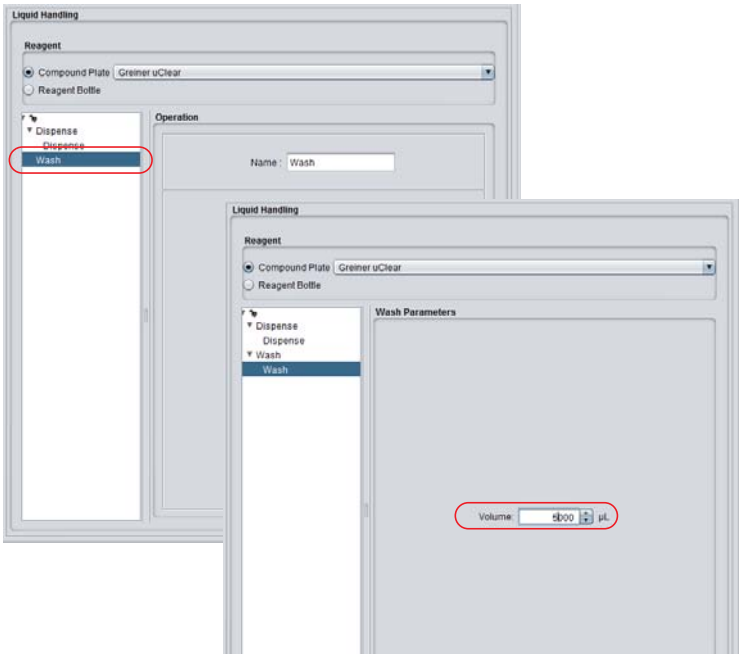
Fig 6-15. Liquid Handling - Dispense add event/parameters



2 Add another operation, ***Wash***, to the Operation tree, by selecting ***Add new operation*** again. Under *Sequence*, mark ***Post experiment*** as you are adding the same Reagent to all wells.

Right click on the ***Wash*** operation and select ***Add new event***. Select the ***Wash*** option from drop down menu. In the *Wash Parameters* panel, set the ***Volume*** to *5000µl* (volume range: 1000-5000µl).

Fig 6-16. Liquid Handling - Wash add event/parameters



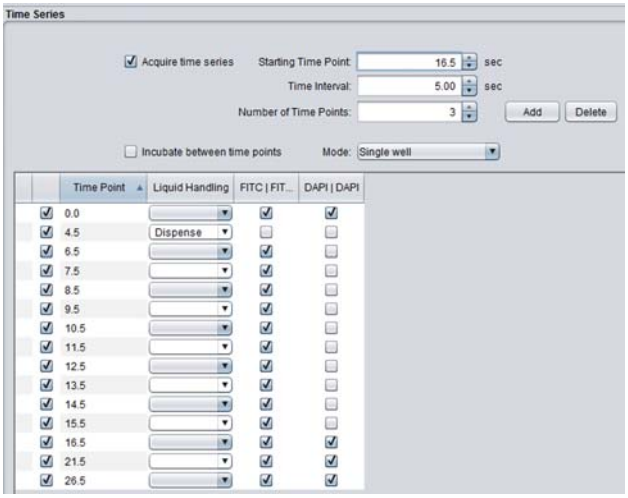
- **Time Series** - Mark ***Acquire time series*** and set ***Mode*** to ***Single well.*** Do not mark ***Incubate between time points*** (used with long time courses not fast kinetic imaging)***.***

  Acquire baseline FITCI and DAPI images at ***Time Point*** *0 sec* and dispense only (no imaging) at ***Time Point*** *4.5 sec*.

  Acquire FITC images at ***Time Point*** *6.5 sec* with ***Time Interval*** *1 sec* (for an additional 10 sec).

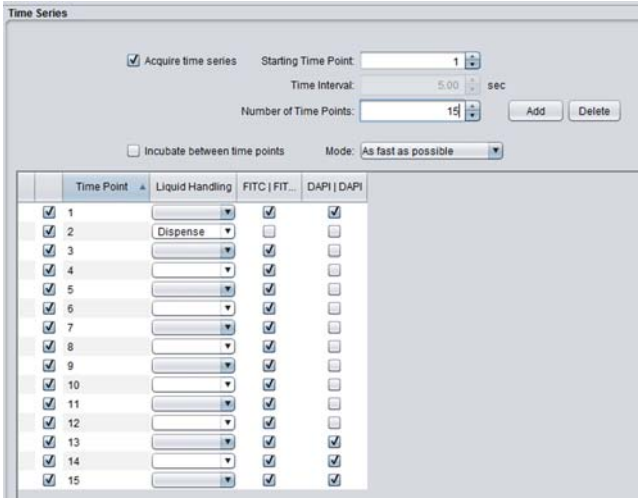
  Acquire FITC and DAPI images at ***Time Point*** *16.5 sec* with ***Time Interval*** *5 sec* to a point where you feel your reaction will be complete.

Fig 6-17. Time series window



- ***As fast as possible*** mode - Input number of time points required. Select at what time point you require a LH operation and select if you require FITC only or FITC and DAPI images. Time interval option is greyed out.

Fig 6-18. Time series window



- **Acquisition options** - Select ***vertical or horizontal serpentine***

  Count Cells and *Batch Analysis* not used in this application.

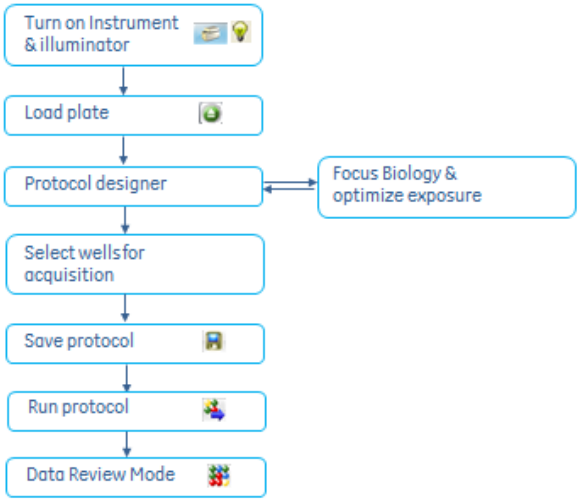
- **Review Scan** is not used in this workflow

7 Image acquisition using liquid handling/multi-well time series

This tutorial describes how to use the ***Liquid Handling*** and ***Time Series*** options to acquire a multi-well time series using a reagent bottle or compound plate with a 30-minute incubation time. A typical workflow is shown in the schematic below.

Note: *The timings used in this tutorial are for guidance only and may not be optimal.*

Fig 7-1. Workflow



Prior to imaging ensure that the biology plate is clean and scratch free.

Turn on the instrument

- Turn on the instrument as described in *Introduction, Turn on the instrument*.

Turn on the Liquid Handling module

- Click the ***Liquid Handling*** toolbar icon to set the wash and reagent bottle volumes, and prime the liquid handling lines prior to use.

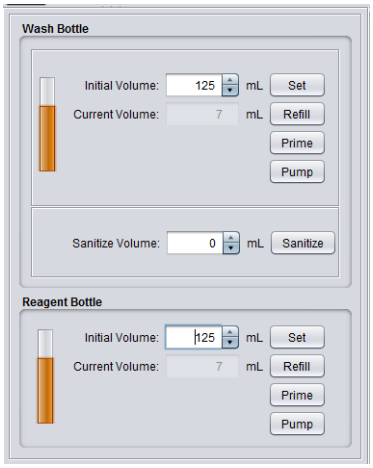
  Ensure lines are clean prior to using liquid handling. If in doubt, wash both lines with sterile water using the ***Prime*** function. The lines can then be washed using

50 or 70% ethanol using the ***Prime*** function followed by 2-3 primes with sterile water to ensure all traces of ETOH have been removed.

On completion of your experiment it is advisable to prime both the wash and reagent lines with sterile water to remove all traces of drugs/buffers. The ***Sanitize*** option can be used to flow a volume of sanitizing/wash solution from the wash bottle to the needle. Manually select the volume of cleaning solution to use (1-5ml) and on completion the ***Air dry wash tubing*** option is enabled**.**

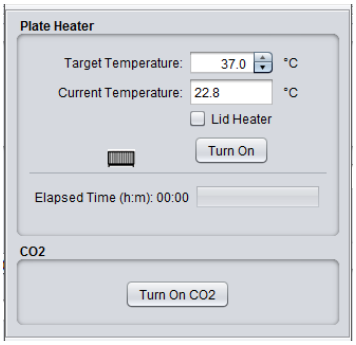
- If using the reagent bottle to dispense compound, the reagent line can be primed for use prior to starting the experiment. The prime volume is 5.5ml and this volume needs to be added to the total volume you need for your experiment.
- If there is insufficient fluid volume to complete an operation the system will prevent execution of the Liquid Handling operations.

Fig 7-2. Liquid handling dialog



- Click on the  ***Environmental Control*** toolbar icon, if plate heating is required and set the ***Target Temperature***. Click the ***Turn On*** button (toggles to ***Turn Off***) to allow the plate heater to reach temperature.

Fig 7-3. Environmental Control



Note: *Lid heater and CO_2 cannot be used with liquid handling.*

Load the plate

- Load the sample and compound plates without lids as described in *Introduction, Load the plate*.

Protocol Designer

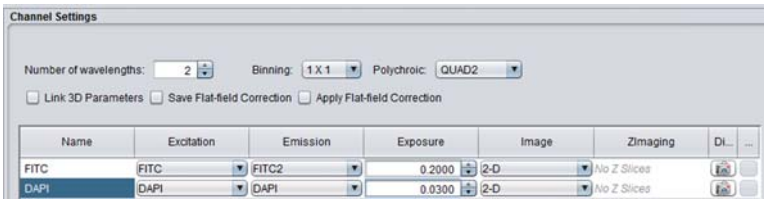
The ***Protocol Designer*** wizard opens at the ***Protocol name*** panel, the default start position for creating a new protocol. Create and optimize an acquisition protocol by navigating the wizard sequentially through each panel. It is not recommended that the ***Dashboard*** feature be used until new users are more familiar with the software (see tutorial 10).

Fig 7-4. Protocol wizard

- **Protocol Name** - Protocol name and description of your experiment.

- **Plate/Slide** - Choose a plate from the drop-down menu (e.g.,Greiner µClear). Plate information available here provides the nominal focus position (Z) at the solid/liquid interface.
- **Objective Lens** - Choose an objective from drop-down menu (e.g., 20× objective), the SAC collar setting is related to the bottom thickness of the plate (e.g. 170µm plate thickness = 0.17 SAC setting).
- **Specimen Options** - Available only when you have selected a slide in the ***Plate/Slide View*** card.
- **Channel Settings** - Select 2 wavelengths as described in *Tutorial 1 Protocol Designer -Channel Settings*. Set *1×1* ***Binning***; ***Polychroic*** QUAD1 or 2; *FITC* and *DAPI* filter pairs (or filters appropriate to your experiment); standard *2D* ***Imaging*** mode; and ***Exposure*** times as shown below.

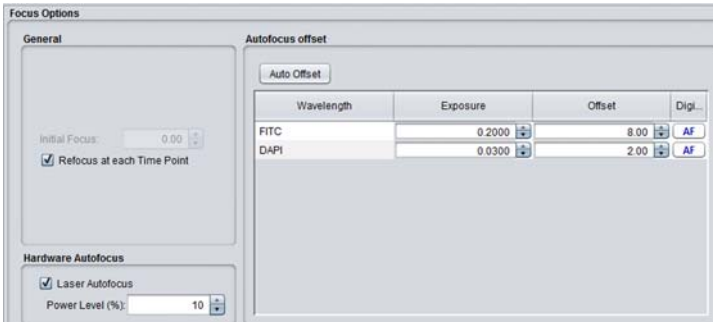
Fig 7-5. Channel Settings card



- **Focus Options** - Select ***Laser Autofocus*** (i.e., hardware autofocus), and then optimize the focus and exposure times at each wavelength.

  The '**Refocus at each Time point'** option will be inactive at this point. Go down to **Time Series** within Protocol Designer and select **Acquire time series**. Go back up to **Focus** and the **Refocus at each Time point'** option will now be active so select it.

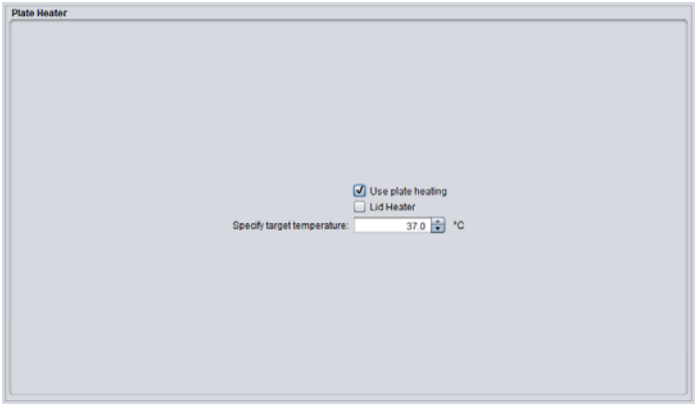
Fig 7-6. Focus card



1. In ***Plate View,*** click within a well where you want to optimize exposure and focus.
2. Click on ***AF*** for *DAPI* wavelength, and visualize the image.
3. In ***Panel View***, open the ***Visuals*** tool by clicking the ***Visuals*** icon.
4. Optimize exposure in each channel by adjusting the exposure time, then pressing ***AF*** button to update the image. Assess exposure by looking at the histogram and *MIN*, *MAX*, and *MEAN* signal strength values.
5. Determine the auto offset by clicking the ***Auto offset*** button. This will provide an offset value for each wavelength.
6. Finally, press the ***AF*** button to update the image. Check grey level ranges in each channel to ensure that the images are not over exposed, and are in focus. The offset position can be adjusted manually, if required.
7. Check the exposure & focus settings in a different well (mouse click in a well) and then image at each wavelength by clicking the ***AF*** button in turn to visualize the new image.

- **Image Processing** not used
- **Plate Heater -** Mark the ***Use plate heating*** checkbox, and set ***Target Temperature*** to 37°C if required. Do not select ***Lid Heater***.

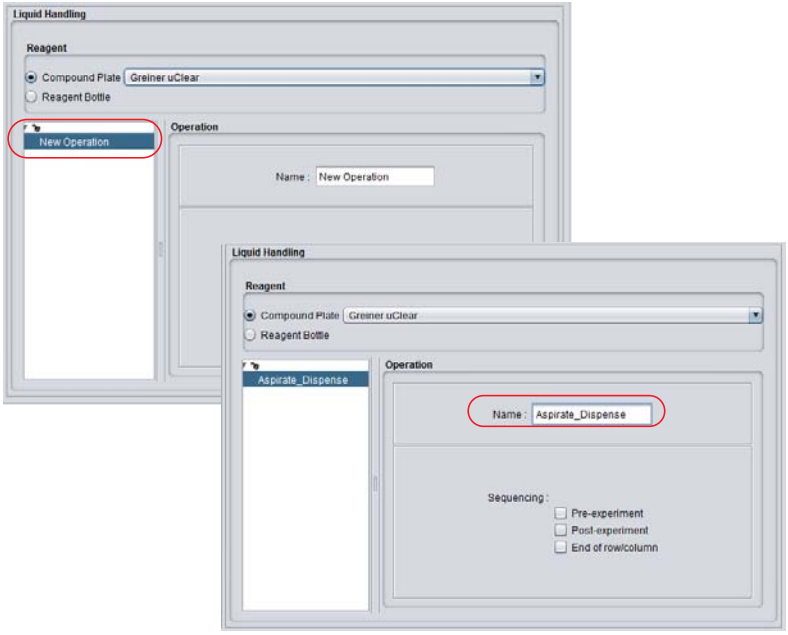
Fig 7-7. Plate Heater card



- **Liquid Handling -** Mark the ***Compound Plate*** radio button and select a plate type from the drop-down list. Ensure the sample plate and compound plates are the same size (i.e., both are 96 well plates).

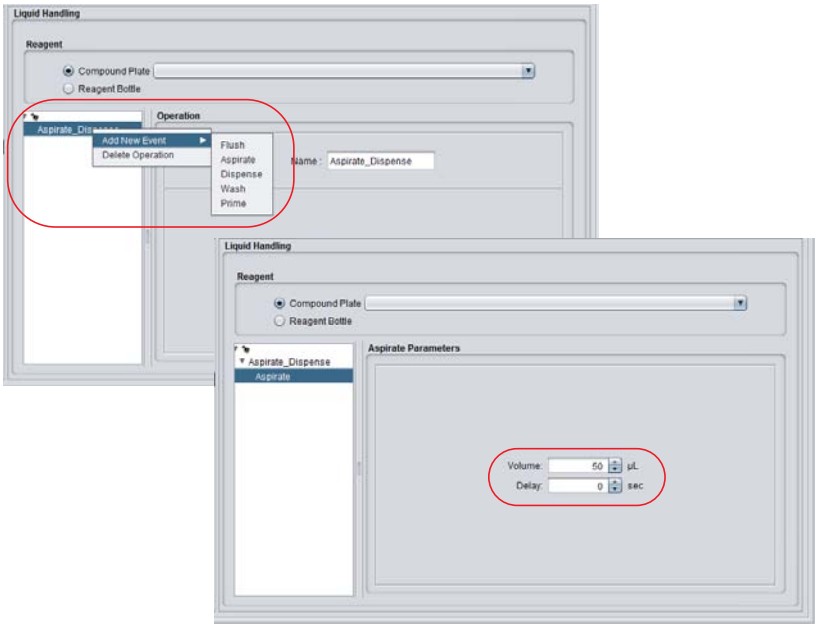
1 In the *Operation* panel, right-click on the root node of the operation tree and select ***Add new operation.*** Name the operation (ASPIRATE_DISPENSE)**.**

Fig 7-8. Liquid Handling - Aspirate_Dispense add operation



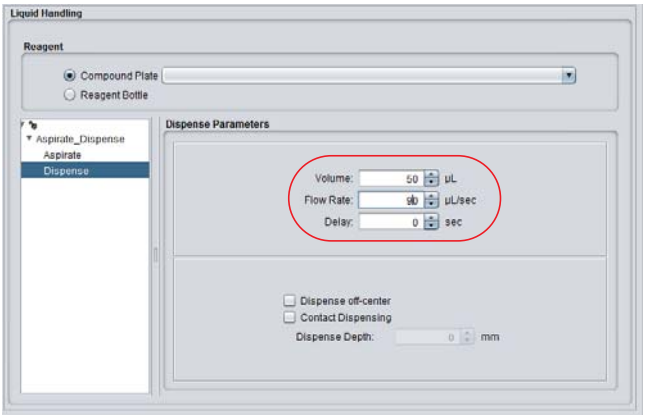
Right-click on the (ASPIRATE_DISPENSE) operation and select the ***Add new event*** option. Select ***Aspirate***.and in the *Aspirate Parameters* panel, set the ***Volume*** to *50µl* (volume range: 10-100µl in steps of 10µl), and the ***Delay*** to *0 sec*. (Note that ***Delay*** is normally used for viscous solutions).

Fig 7-9. Liquid Handling - Aspirate add event/parameters



Within the ASPIRATE_DISPENSE operation add a second event, ***Dispense***, from the ***Add new event*** drop down menu.

Fig 7-10. Liquid Handling - Dispense add event/parameters .



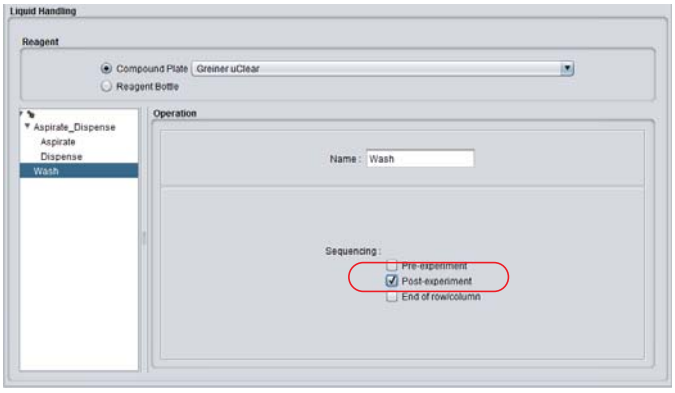
In the *Dispense Parameters* panel enter the values below. Ensure the ***Aspirate*** and ***Dispense*** volumes are equal for this example. (In this protocol

we are dispensing into the center of the well using non-contact dispensing.)

Volume	*50µl* (volume range: 10-100µl in steps of 10µl)
Flow Rate	*90µl/sec* (flow rate range: 50-200µl and is cell type dependent)
Delay	*0 sec*
Dispense off-center	*off*
Contact Dispensing	*off*

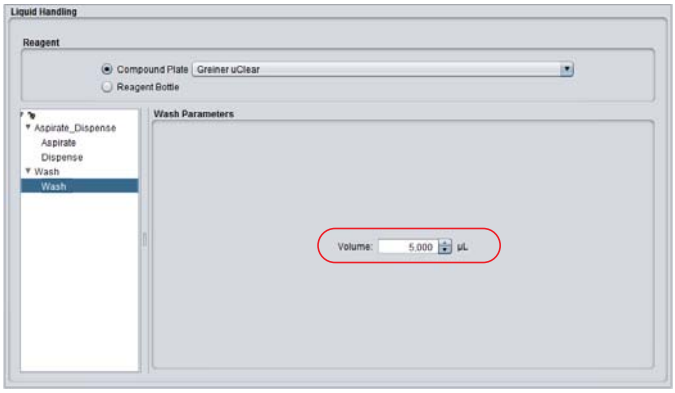
2 Add a second operation, ***Wash***, to the Operation tree, by selecting ***Add new operation*** again. Under *Sequence*, mark ***Post experiment*** as in this example we are adding a dose response curve from compound plate to sample plate going from lowest to highest concentration. You would also select this option if you were adding the same compound to all wells from compound plate or when using reagent bottle. Minimizing wash steps allows for faster acquisition.

Fig 7-11. Liquid Handling - Wash add operation/post experiment option .



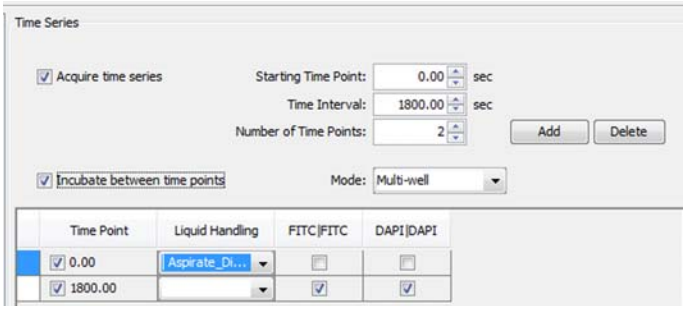
Right-click on the ***Wash*** operation, select the ***Add new event***, and select ***Wash***. In the *Wash Parameters* panel, set the ***Volume*** to *5000µl* (volume range: 1000-5000µl).

Fig 7-12. Liquid Handling - Wash add event/parameters .



- **Time Series**: - The ***Acquire time series*** checkbox was previously marked**.** Set ***Mode*** to ***Multi-well*** and mark the ***Incubate between time points*** checkbox if using plate heating (to return plate to incubate position after the liquid handling event).

Fig 7-13. Time series window



  - Set the ***Starting Time Point*** to *0 sec*; set the ***Time Interval*** to *1800 sec*; set the ***Number of Time Points*** to *2*. Click ***Add***.

  - For ***Time Point*** *0.0,* select *Aspirate_Dispense* from the ***Liquid Handling*** drop-down list and unmark the *FITC* and *DAPI* checkboxes as we do not want to acquire images at this time point.

  - At ***Time Point*** *1800 secs* (30 mins), FITC and DAPI images will be obtained exactly 30 minutes after the dispense operation.

- **Acquisition options**: - Select ***vertical Serpentine*** (if adding a low-high dose-response curve from compound plate).

  Count Cells and *Batch Analysis* not used in this example.

- **Review Scan** is not used in this workflow

Select wells for acquisition

In ***Plate View***, window, the yellow-colored wells are marked for acquisition. To *deselect wells* use **CTRL** | **SHIFT** | **LEFT CLICK** and drag. To *select wells* use **CTRL** | **LEFT CLICK** and drag.

Save & run the protocol 

- Save the protocol as described in *Introduction, Save the protocol.*
- Run the protocol as described in *Introduction, Run the protocol.*
- ***Data Review mode*** will open automatically to review the post acquisition image stacks as described in *Introduction, Data Review mode.*

Shutting down the instrument 

- Turn off the instrument as described in *Introduction, Shutting down the instrument*.

Appendix: Using the reagent bottle

Using Reagent bottle (example: - when adding agonist to all wells that may have been treated with an antagonist dose response curve).

Turn on the Liquid Handling module 

- Turn on the ***Liquid Handling*** module as described at the start of this tutorial.

Create Protocol

Create and optimize an acquisition protocol using the ***Protocol Designer***. Navigate through the wizard using the information given earlier in this chapter up to ***Focus Options***.

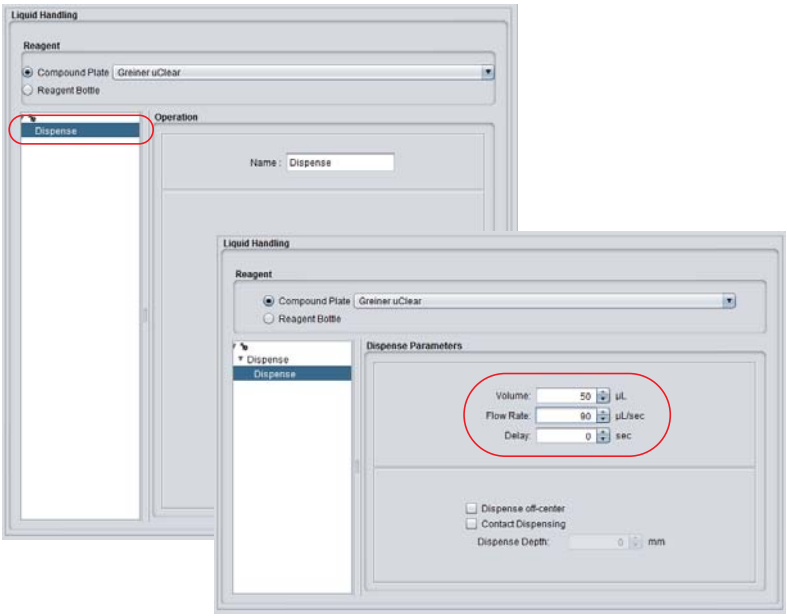
- **Focus Options** - Ensure ***Refocus between Time points*** is selected.
- **Liquid handling -** Mark the ***Reagent bottle*** radio button.

1 In the *Operation* panel, right-click on the root node of the operation tree and select ***Add new operation.*** Name the operation ***Dispense***. Right click on the

Dispense operation and select ***Add new event***. Select ***Dispense*** from the drop down menu.

2 In the *Dispense Parameters* panel, set the ***Volume*** to *50µl*, set the ***Flow Rate*** to *90µl/sec* (for example), and the ***Delay*** to *0 sec*.

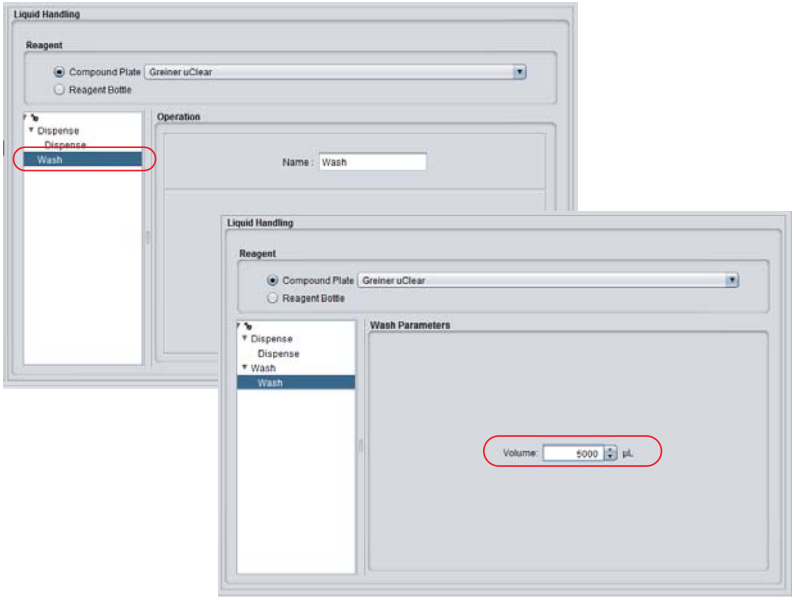
Fig 7-14. Liquid Handling - Dispense add event/parameters .



3 Add another operation, ***Wash***, to the Operation tree, by selecting ***Add new operation*** again. Under *Sequence*, mark ***Post experiment*** as you are adding the same compound to all wells. Minimizing wash steps allows for faster acquisition.

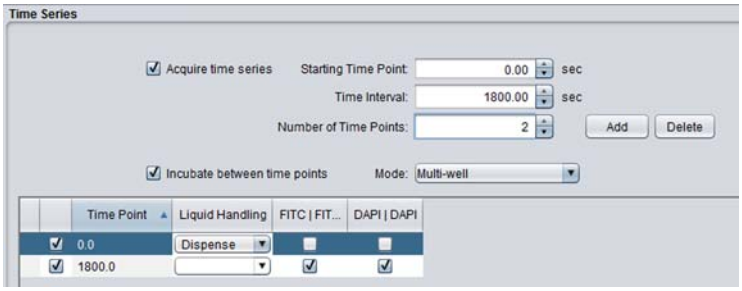
Right-click on the ***Wash*** operation, select the ***Add new event***, and select ***Wash***. In the *Wash Parameters* panel, set the ***Volume*** to *5000µl* (volume range: 1000-5000µl).

Fig 7-15. Liquid Handling - Wash add event/parameters



- **Time Series** - Mark ***Acquire time series*** and set ***Mode*** to ***Multi-well.*** Mark the ***Incubate between time points*** checkbox if using plate heater.

Fig 7-16. Time series window



- Set the ***Starting Time Point*** to *0 sec*; set the ***Time Interval*** to *1800 sec*; set the ***Number of Time Points*** to *2*. Click ***Add***.
- For ***Time Point*** *0.0,* select ***Dispense*** from the ***Liquid Handling*** drop down menu and unmark the FITC and DAPI checkboxes as we do not want to acquire images at this time point.
- At ***Time Point*** *1800 secs* (30 mins), FITC and DAPI images will be obtained exactly 30 minutes after the dispense operation.

Note: *If you want to acquire images prior to dispensing at Time Point 0.0 tick the*

FITC and DAPI checkboxes and ensure no Liquid Handling options are selected from the drop down list
For Time Point 300.0 secs, select Dispense from the Liquid Handling drop down menu and unmark the FITC and DAPI checkboxes as we do not want to acquire images at this time point
For Timepoint 2100.0 secs., mark the FITC and DAPI checkboxes so images are acquired 30 minutes after addition of agonist.

- **Acquisition options** - Select ***Horizontal*** or ***Vertical Serpentine***

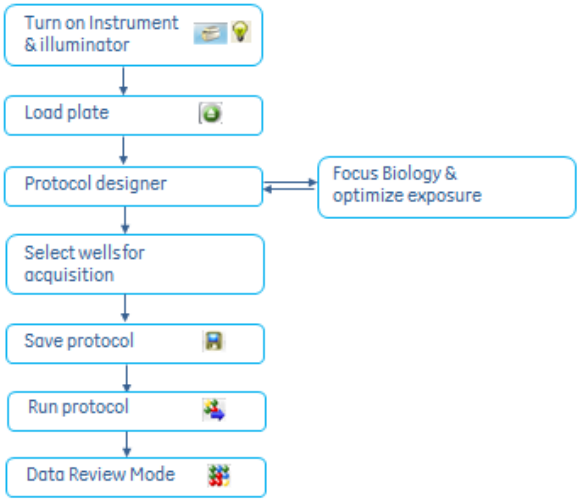
  Count Cells and *Batch Analysis* not used in this application.

- **Review Scan** is not used in this workflow.

8 Image acquisition using environmental control/multi-well time series

This tutorial describes how to acquire a 72-hour multi-well extended time series using Environmental Control and imaging every four hours. A typical workflow is shown in the schematic below.

Fig 8-1. Workflow



Prior to imaging ensure that the biology plate is clean and scratch free and is sealed with a Breatheasy plate sealer.

Turn on the instrument & illuminator

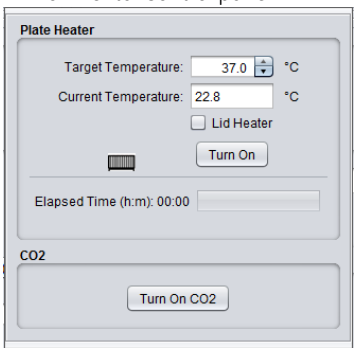
- Turn on the instrument as described in *Introduction, Turn on the instrument*.
- Turn on the lamp from the toolbar ***Illuminator*** icon as described in *Introduction, Turn on the instrument*.
- Turn on the CO_2 supply to the instrument.

Turn on Environmental Control

Click on the ***Environmental Control*** toolbar icon to open the Environmental Control window.

- In the *Plate Heater* panel, mark the ***Lid Heater*** checkbox and set the ***Target Temperature***. Click the ***Turn On*** button (toggles to ***Turn Off***) to allow the plate heater to reach temperature.
- In the *CO2* panel, click ***Turn On CO2***. Ensure CO_2 is bubbling through the bottle.

Fig 8-2. Environmental Control panel



Load the plate

- Load an empty plate without its lid and cover with the environmental control chamber lid.
- Connect the lid heater electrical connector and the CO_2 tubing to the environmental control chamber lid.
- Close the door by clicking on any well shown in the ***Plate View***. Leave the instrument to equilibrate for ~60 minutes before use.
- When the system is ready, remove the empty plate and replace with the sample/test plate (with its lid removed and sealed with a Breatheasy membrane) that has been equilibrated to 37°C/5% CO_2 in a tissue culture incubator prior to use on the instrument.
- Cover the plate with the environmental control chamber lid with the electrical connector and CO_2 tubing connected.

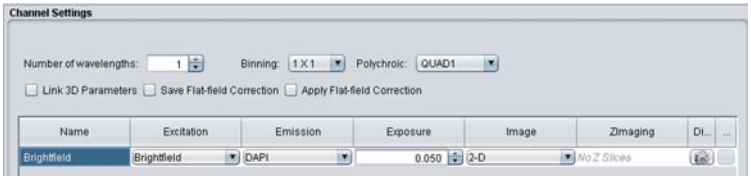
Protocol Designer

The ***Protocol Designer*** wizard opens at the ***Protocol name*** panel, the default start position for creating a new protocol. Create and optimize an acquisition protocol by navigating the wizard sequentially through each panel. It is not recommended that the ***Dashboard*** feature be used until new users are more familiar with the software (see tutorial 10).

Fig 8-3. Protocol wizard

- **Protocol Name** - Protocol name and description of your experiment.
- **Plate/Slide** - Choose a plate from the drop-down menu (e.g., Greiner µClear). Plate information available here provides the nominal focus position (Z) at the solid/liquid interface.
- **Objective Lens** - Choose an objective from drop-down menu (e.g., 20× objective), the SAC collar setting is related to the bottom thickness of the plate (e.g. 170µm plate thickness = 0.17 SAC setting).
- **Specimen Options** - Available only when you have selected a slide in the ***Plate/Slide*** card.
- **Channel Settings** - Select 1 wavelength as described in *Tutorial 1 Protocol Designer -Channel Settings*. Set *1×1* ***Binning***; ***Polychroic*** QUAD1; Bright field/ DAPI excitation/emission; standard *2D* ***Imaging*** mode; and ***Exposure*** times as shown below.

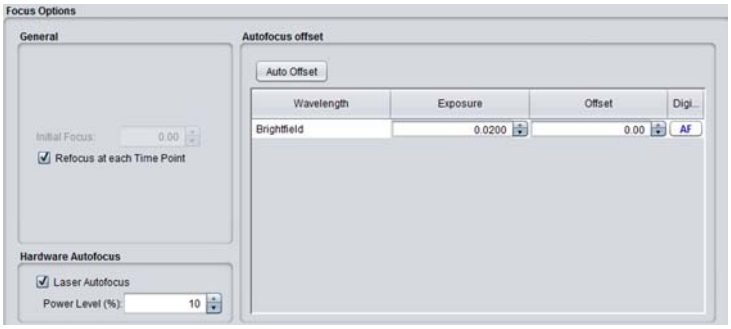
Fig 8-4. Channel Settings card



- **Focus Options** - Select ***Laser Autofocus*** (i.e., hardware autofocus), and then optimize the focus and exposure times at each wavelength.

  The ***Refocus at each Time point*** option will be inactive at this point. To activate this option go down to ***Time Series*** in the Protocol Designer and select ***Acquire time series***. Go back up to ***Focus*** and the ***Refocus at each Time point*** option will now be active so select it.

Fig 8-5. Focus card



1. In ***Plate View,*** click within a well where you want to optimize exposure and focus.
2. Click on ***AF*** for *Brightfield* wavelength, and visualize the image.
3. In ***Panel View***, open the ***Visuals*** tool by clicking the ***Visuals*** icon.
4. Optimize exposure in each channel by adjusting the exposure time, then pressing ***AF*** button to update the image. Assess exposure by looking at the histogram and *MIN*, *MAX*, and *MEAN* signal strength values displayed at the bottom of the ***Panel View***.
5. Determine the auto offset by clicking the ***Auto offset*** button. This will provide an offset value for each wavelength.

   When using transmitted light it is common to have to set the offset manually as the auto offset uses a contrast based algorithm to find the focus position and therefore will find the brightest spot. This may not be

optimal for a bright field image where there may be poor contrast between the cell and the background.

6 Finally, press the ***AF*** button to update the image. Check grey level ranges in each channel to ensure that the images are not over exposed, and are in focus. The offset position can be adjusted manually, if required.

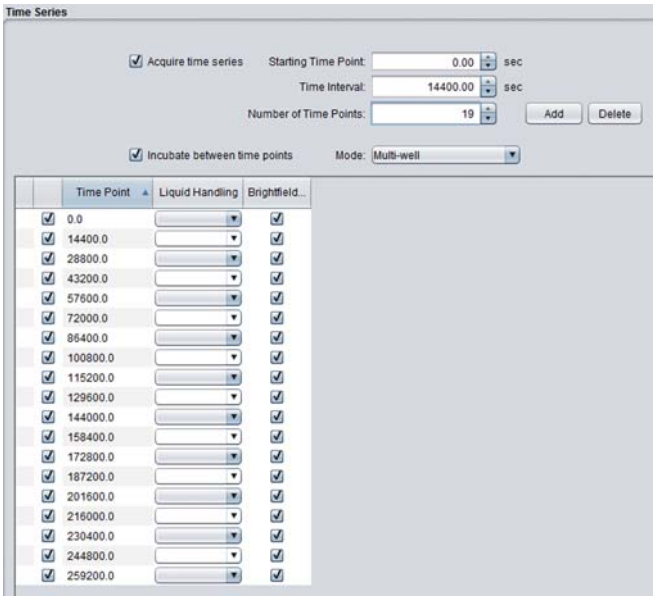
7 Check the exposure & focus settings in a different well (mouse click in a well).

- **Image Processing** not used
- **Plate Heater -** Mark the ***Use plate heating*** and ***Lid Heater*** checkboxes, and set ***Target Temperature***.

Fig 8-6. Plate Heater card

- **Liquid handling-** cannot be used with Environmental Control.
- **Time Series** - The ***Acquire time series*** checkbox was previously marked**.** Set ***Mode*** to ***Multi-well*** and mark the ***Incubate between time points*** checkbox.

Fig 8-7. Time series window



- Set the ***Starting Time Point*** to *0.0 sec*; set the ***Time Interval*** to *14400 sec*; set the ***Number of Time Points*** to *19*. Click ***Add***.

- **Acquisition options**: - Select ***Horizontal Serpentine*** (any other option can be used).

  Count Cells and *Batch Analysis* not used in this example.

- **Review Scan** is not used in this workflow

Select wells for acquisition

In ***Plate View***, window, the yellow-colored wells are marked for acquisition. To *deselect wells* use **CTRL**|**SHIFT**|**LEFT CLICK** and drag. To *select wells* use **CTRL**|**LEFT CLICK** and drag.

Save & run the protocol

- Save the protocol as described in *Introduction, Save the protocol*.
- Run the protocol as described in *Introduction, Run the protocol*.
- ***Data Review mode*** will open automatically on completion of the time series to review the post acquisition image stacks as described in *Introduction, Data Review mode*.

Shutdown the Environmental Control 

- Click the ***Environmental Control*** toolbar icon, unmark the lid heater checkbox, and click the ***Turn Off*** button (toggles to ***Turn On***) for both temperature and CO_2.
- Eject the plate from instrument and disconnect the Environmental Control lid from both the electrical connector and the CO_2 tubing. Remove plate from stage taking care not to touch the stage as it may still be warm.
- Turn off the main CO_2 supply to the instrument.

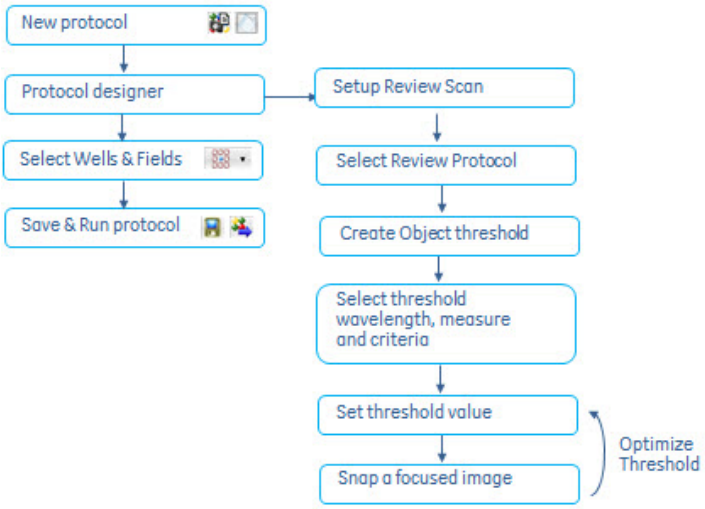
Shutting down the instrument 

- Turn off the instrument as described in *Introduction, Shutting down the instrument*.

9 Use of Review Scan

This tutorial describes the use of the Review Scan feature to select wells, acquired on a typical biology plate, based on cellular measure criteria that will then be analyzed with another protocol. Some wells in the sample 96 well plate (EGFP-2 X FYVE) have been spiked with DRAQ5, as a nuclear stain. For the review scan using Object Thresholding, a new protocol is written to scan the 96 well plate with a 10X objective (lower magnification) to identify the DRAQ5 +ve wells. Wells identified are automatically rescanned at higher magnification (20X) using the protocol created in Tutorial 1. A typical workflow is shown in the schematic below.

Fig 9-1. Workflow



Create review scan protocol

- Ensure you are in ***Assay Development mode.*** If not click on the toolbar icon to return to ***Assay Development*** mode, and select ***File|New*** or click on the ***Start a new protocol*** icon to create a new protocol.

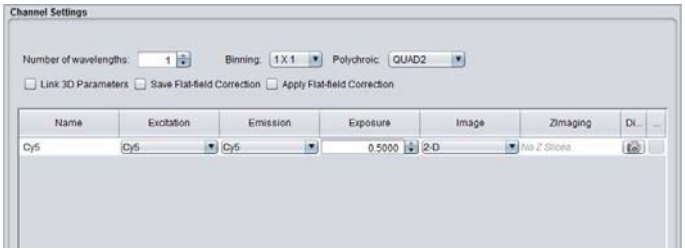
Protocol Designer

The ***Protocol Designer*** wizard opens. Create and optimize an acquisition protocol by navigating the wizard sequentially through each panel. It is not recommended that the ***Dashboard*** feature be used until new users are more familiar with the software (see tutorial 10).

Fig 9-2. Protocol wizard

- **Protocol Name** - Protocol name and description of your experiment.
- **Plate/Slide** - Choose a plate from the drop-down menu (e.g., Greiner µClear).
- **Objective Lens** - Select the 10X objective.
- **Specimen Options** - Available only when you have selected a slide in the ***Plate/Slide*** card.
- **Channel Settings** - Select 1 wavelength (e.g., CY5) to apply the threshold for well selection as described in *Tutorial 1 Protocol Designer -Channel Settings*. Additional wavelengths can also be selected if required. Set *1×1* ***Binning***; *QUAD2* ***Polychroic***; standard *2D* ***Imaging*** mode; and nominal ***Exposure*** times as shown

Fig 9-3. Channel Settings card



- **Focus Options** - Select ***Laser Autofocus*** (Hardware Autofocus), focus the biology and adjust exposure and set auto offset for each channel as described in tutorial 1.
- **Image Processing**, **Plate Heater, Liquid handling, and Time Series** are not used in this workflow

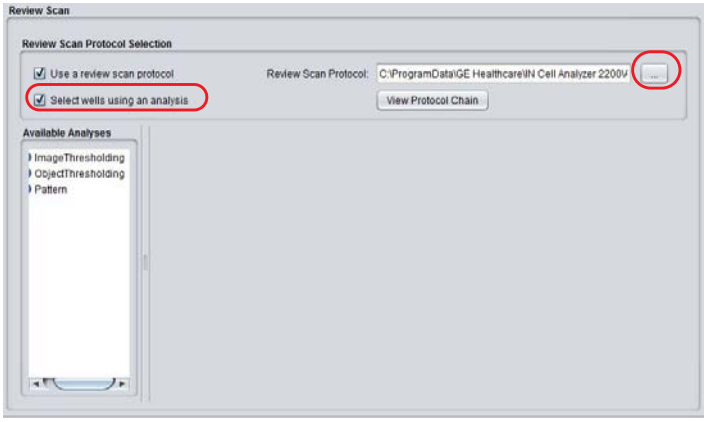
- **Acquisition options**: - Select ***Horizontal Serpentine***.

  Count Cells and *Batch Analysis* not used in this example.

- **Review Scan** - Check the ***Use a review scan protocol option*** checkbox and click the R***eview Scan Protocol*** field ***Browse*** button to select the protocol created in Tutorial 1.

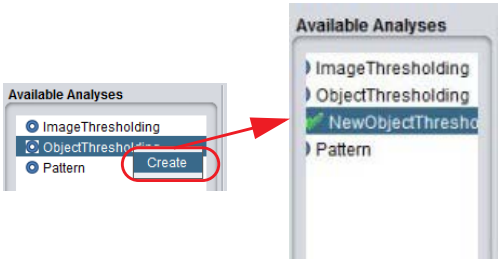
1 Check ***Select wells using an analysis*** checkbox. The ***Available Analyses*** list is enabled.

Fig 9-4.



2 Select and right-click on ***ObjectThresholding*** and then click ***Create*** to create the ***NewObjectThresholding*** entry.

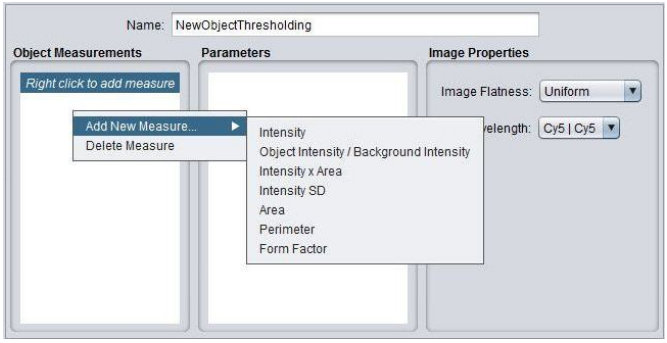
Fig 9-5.



3 In the ***Object Measurements*** window select A***dd New Measures*** from the right-click menu to add your thresholding measures. Select Intensity.

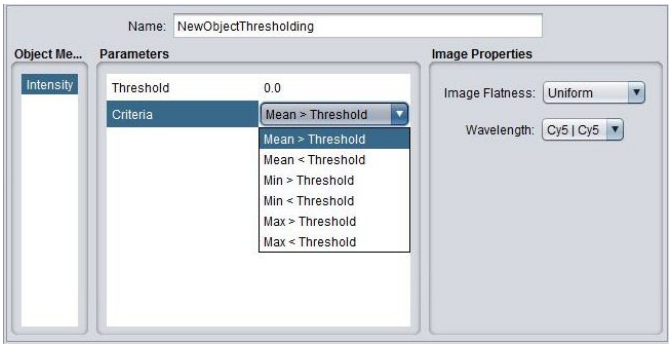
Note: *If more than one measure is selected for thresholding then only those wells that meet all the threshold criteria would be selected*

Fig 9-6.



4 In the ***Parameters*** window click ***Mean > Threshold*** from the ***Criteria*** dropdown box. Only the wells with Intensity (Mean) greater than the threshold value defined for ***Threshold*** for the selected wavelength will be selected. DO not modify the ***Threshold*** value.

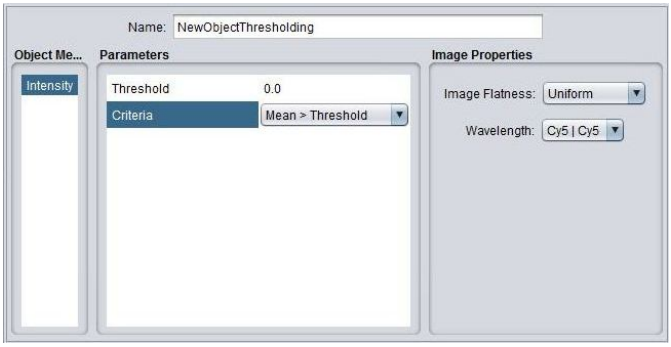
Fig 9-7.



5 Under Image Properties, select the ***Wavelength*** to be used to set the threshold and select ***Non-uniform*** from the ***Image Flatness*** dropdown list.

Note: *Select Uniform for image flatness if there are no large intensity variations in the image or no bright spots.*

Fig 9-8.



6 Select the ***Focus Options*** from the Protocol Designer wizard. Select a well spiked with DRAQ5 (+ve well) to image. Click the ***Digitize/AF*** button in the Cy5 channel. The segmented Image is displayed in the ***Panel View*** window. The Intensity (Mean) value of the segmented image displays below the image. Record the value.

Fig 9-9.

7 Capture an image and record the Intensity (Mean) value in a well where DRAQ5 is absent (-ve well).

Repeat in a few more +ve and –ve wells.

8 Select ***Review Scan*** from the Protocol Designer wizard. In the ***Parameters*** window, enter a threshold value between the Intensity (Mean) values recorded for the +ve and –ve wells.

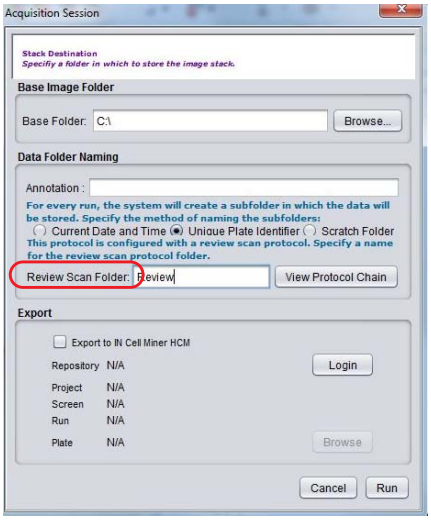
Select field for acquisition

- Go to ***Plate View*** and click on ***Fields to acquire*** icon.
- Acquire one field at the center of the well
  - change the number of fields to one
  - click the re-center button
- Select wells for acquisition as described previously in tutorial 1

Save & run the protocol

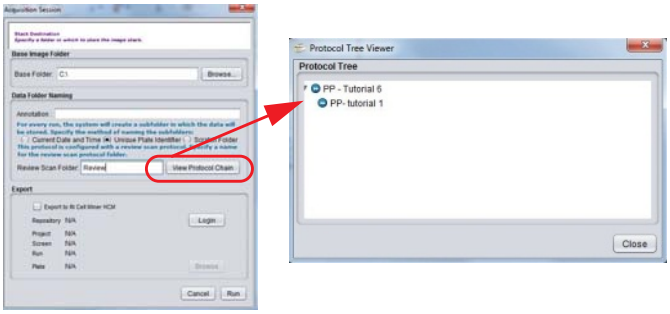
- Save the protocol as described in *Introduction, Save the protocol.*
- Run the protocol as described in *Introduction, Run the Protocol.* Specify a ***Review scan folder*** where all the data, current and review scan output, is saved within the ***Base Folder***.

Fig 9-10.



Note: *Click the* ***View Protocol Chain*** *button to view all the protocols that will*

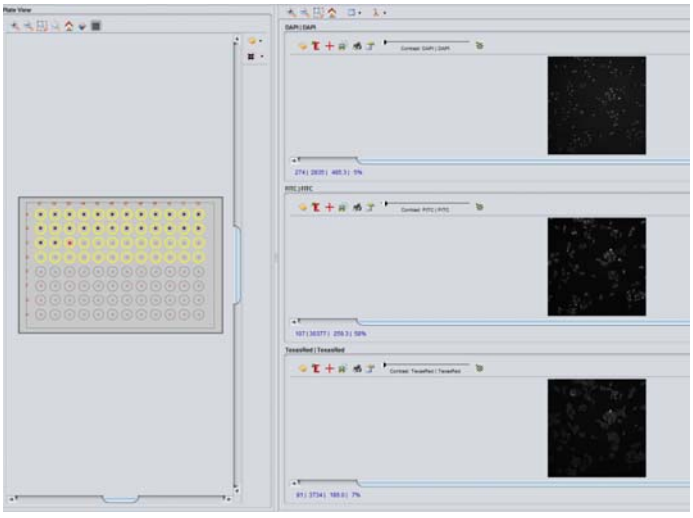
be run in the Review Scan sequence (i.e., protocol chain).



Data Review

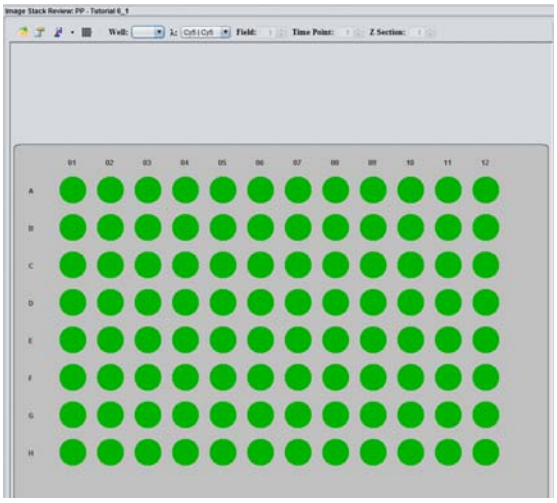
- On completion of the scan, the acquired data is:
  - loaded to the Data Review page
  - Image analysis using the Object Thresholding method begins.
  - A progress bar displays during the image analysis in the lower right corner.
- Upon completion of the image analysis, wells that meet the Cy5 channel Intensity (Mean) > threshold display in the ***Plate View*** window. The plate is now scanned with the review scan (high magnification) protocol.

Fig 9-11.



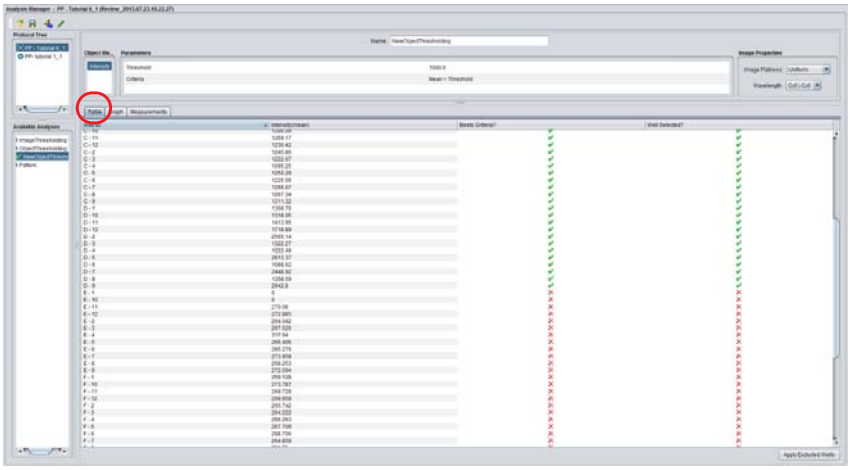
- When the review scan run completes, the results of the low magnification (first scan) display in ***Data Review*** mode. Double-click on individual wells to review data

Fig 9-12.



- Click the ***Analysis mode*** main toolbar icon. The ***Analysis Manager*** window opens. The wells selected for review scan and measure values are displayed. The ***Table*** tab opens by default.

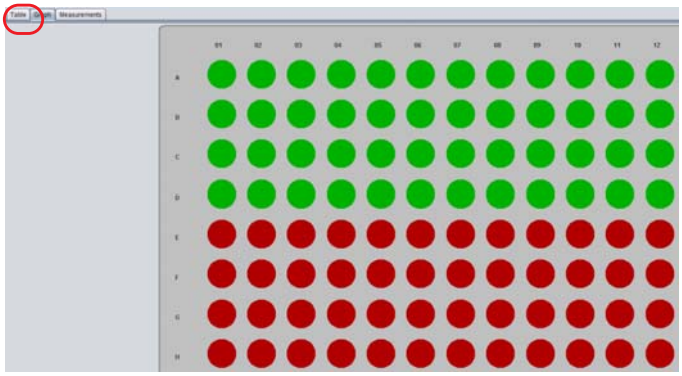
Fig 9-13.



Note: *The data table can be exported as a CSV file for further analysis. Right-click anywhere within the data table and select* ***Export as CSV****.*

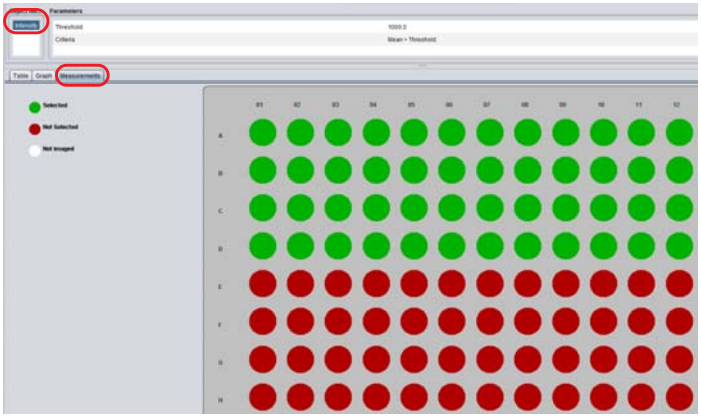
- Open the ***Graph*** tab to view the plate map showing the wells that meet the object threshold criteria.

Fig 9-14.



- Select the ***Measurements*** tab to view the wells meeting the parameters of the threshold criteria. Click on the measures in the ***Object Measure*** panel to show the wells associated with that measure.

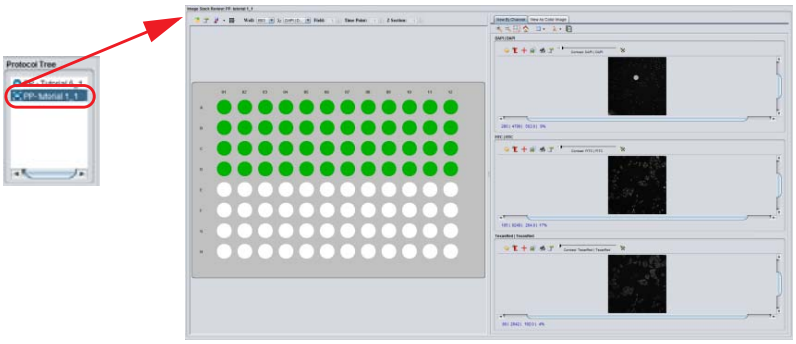
Fig 9-15.



- To view the review scan protocol data, select the review scan protocol from the ***Protocol Tree*** panel in the upper left of the ***Analysis Manager*** window and then select the ***Data Review mode*** toolbar icon.

  Double-click on an acquired well to view the review scan data.

Fig 9-16.

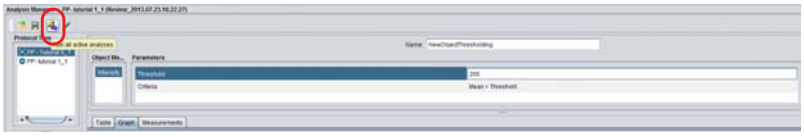


Change the thresholds & reanalyze the data

Threshold values and criteria, as well as the addition of new measures for well selection can be done post-acquisition. The new set of selected wells can then be rescanned.

- To change threshold value, open ***Analysis mode*** from the main toolbar. Double-click on the threshold value and enter the new value. Click on the ***Run analyses*** icon on the ***Analysis Manager*** toolbar to reanalyze.

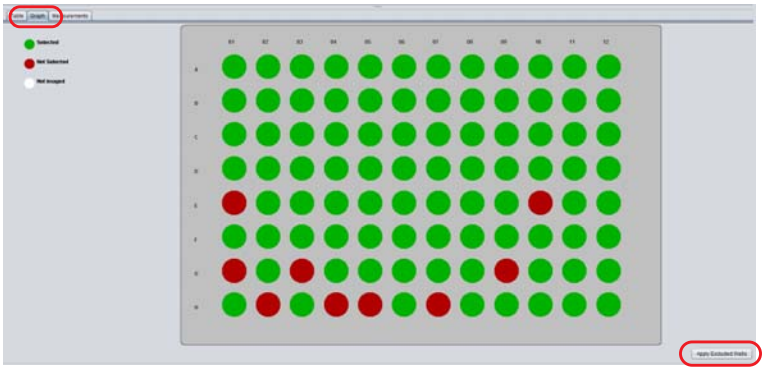
Fig 9-17.



A progress bar displays during the image analysis in the lower right corner of the ***Analysis Manager*** window.

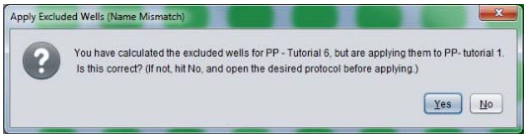
- After re-analysis, the wells selected using the new threshold criteria display on the ***Graph*** tab.

Fig 9-18.



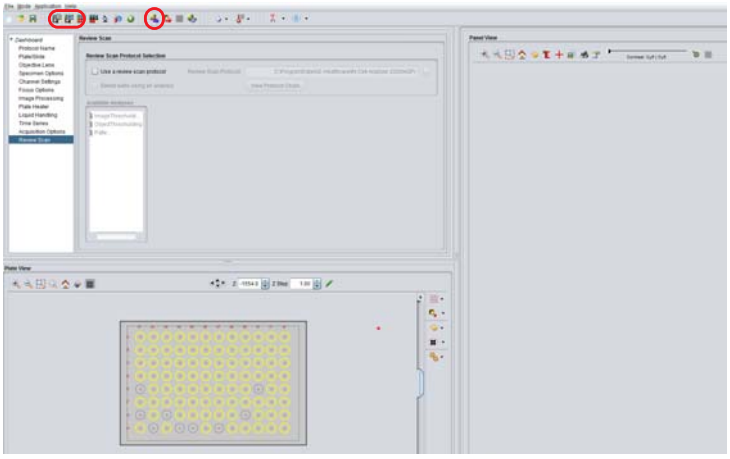
- Rescan the new set of wells with the review scan (high magnification) protocol, click the ***Apply excluded wells*** button. Verify that the wells to be excluded are applied to the correct protocol and click ***Yes*** to the following message.

Fig 9-19.



- Open either ***Acquisition*** or ***Assay Development*** mode from the main toolbar. The new set of wells displays in the ***Plate View*** window. Click the ***Run protocol*** icon on the main toolbar to rescan.

Fig 9-20.



Shutting down the instrument 

- Turn off the instrument as described in *Introduction, Shutting down the instrument*.

10 Using Dashboard to image a typical biology plate

This tutorial describes the use of the ***Dashboard*** feature to image a typical biology plate (e.g., plate used in Tutorial 1). The Dashboard allows the confident user to create and modify protocols without navigating the Protocol Designer wizard for standard plate imaging.

Create protocol

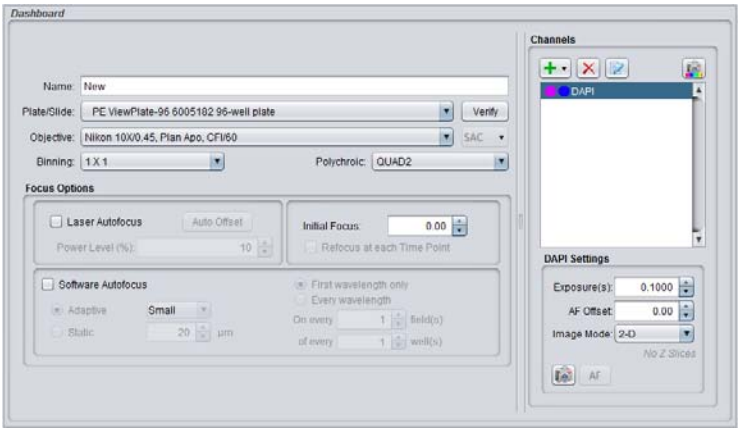
Select ***Dashboard*** from the wizard if not set as the default.

Note: *Dashboard can be set as default from* ***File|Preferences*** *and then selecting Dashboard as the* ***Start Page*** *on the* ***User*** *tab.*

Fig 10-1. Protocol wizard

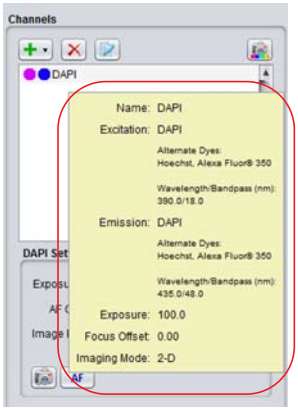
On the ***Dashboard*** panel, create a new protocol.

Fig 10-2.



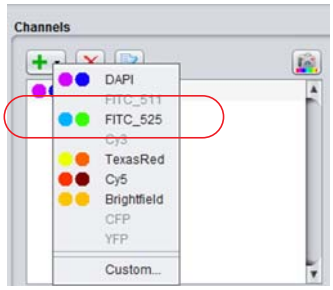
- **Name** - Provide a name for your experiment.
- **Plate/Slide** - Choose a plate from the drop-down menu. Plate information available here provides the nominal focus position (Z) at the solid/liquid interface.
- **Objective** - Choose an objective from drop-down menu.
- **Binning –** Select binning options from drop down menu (e.g., 1x1).
- **Polychroic –** Select polychroic from drop down menu (e.g., QUAD 2)
- **Focus Options** - Mark the ***Laser Autofocus*** checkbox. ***Software Autofocus*** can be selected if required.
- **Channels –** DAPI is selected by default. Hold the mouse over *DAPI* to see the channel information about *Excitation/Emission* filter and alternative dye options.

Fig 10-3.



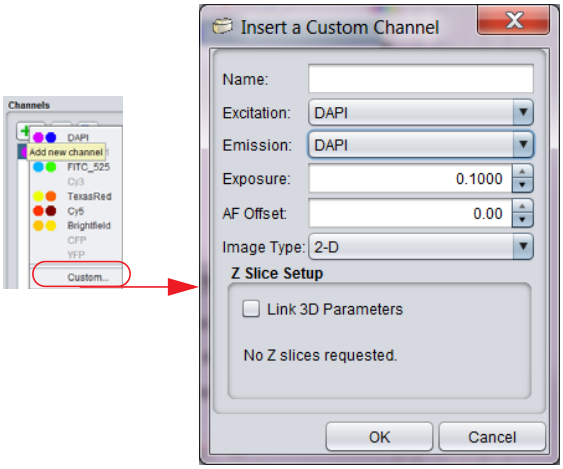
To add channels, click the ***Add Channel*** icon . Add channels appropriate to your experiment.

Fig 10-4.



- Use the ***Custom*** option to create custom channels with the desired Excitation-Emission combination.

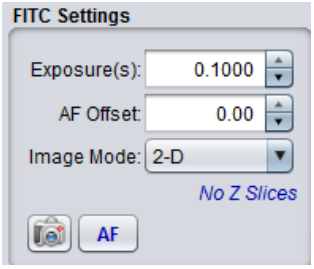
Fig 10-5.



- Channels can be deleted from the list by clicking the ***Remove Channel*** icon or modified by clicking the ***Edit channel*** icon after highlighting the channel.

- **Channel Settings** - Click on each of the channels you have added to the list to display the ***Exposure***, ***AF Offset***, and ***Imaging*** mode. Use the dropdown lists to modify the settings.

Fig 10-6.



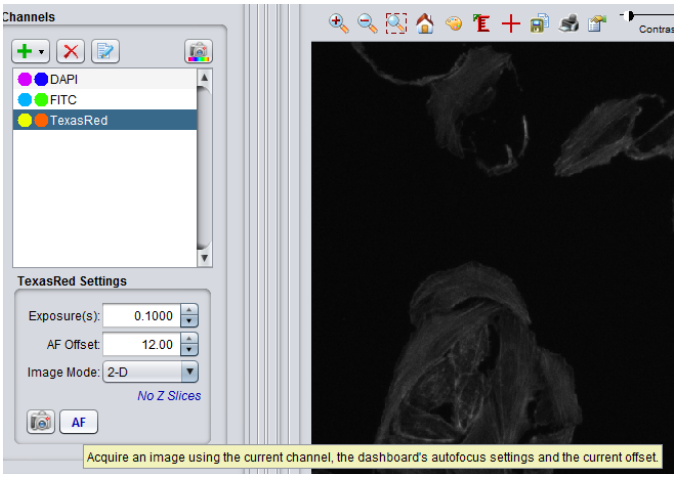
- Double-clicking on a channel name will perform an autofocus and snap an image. Optimize exposure time such that the captured image intensity is not saturated, as described in tutorial 1.

  Single-clicking on a channel will capture an image without applying autofocus.

After optimizing exposure times for all channels, get an offset value for each wavelength by clicking ***Auto offset*** in the ***Focus Options*** panel. Single-click on a

channel name (e.g., Texas Red) and click ***AF*** to see focus with offset added. Repeat to optimize the focus in other wells.

Fig 10-7.



Select wells for acquisition

In ***Plate View***, window, the yellow-colored wells are marked for acquisition. To *deselect wells* use **CTRL**|**SHIFT**|**LEFT CLICK** and drag. To *select wells* use **CTRL**|**LEFT CLICK** and drag.

Save & run the protocol

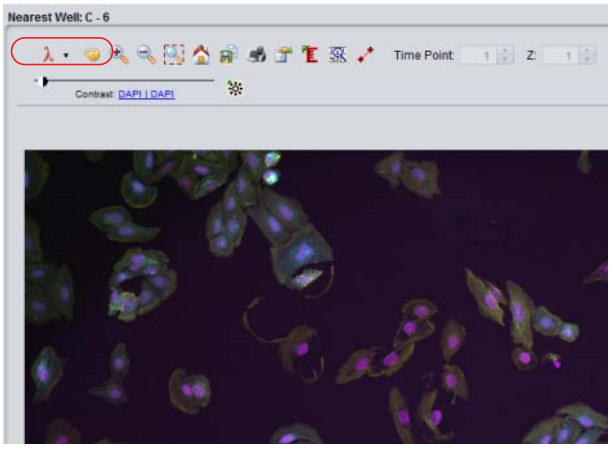
- Save the protocol as described in *Introduction, Save the protocol*.
- Run the protocol as described in *Introduction, Run the protocol*.

Acquiring & saving a color image

The ***Dashboard*** can also be used to acquire a color image using the ***Acquire color image*** option.

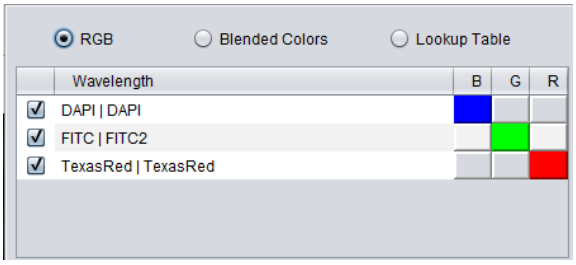
- Optimize the ***Exposure*** and ***Focus*** of the individual channels (as described above) and click the ***Acquire color image*** button in the ***Channels*** panel. A colored (fused) image will display in the ***Panel View*** window.

Fig 10-8.



- In the ***Panel View*** window, click the ***Wavelength*** toolbar icon to change the color options and the ***Visuals*** toolbar icon to adjust the contrast for each wavelength.

Fig 10-9.



- To save the colored image, click on  icon. The image is saved as a *JPEG* file.

Appendix A Specifications

A.1 Polychroic profiles

Fig 10-10. QUAD 1 Polychroic [Y]

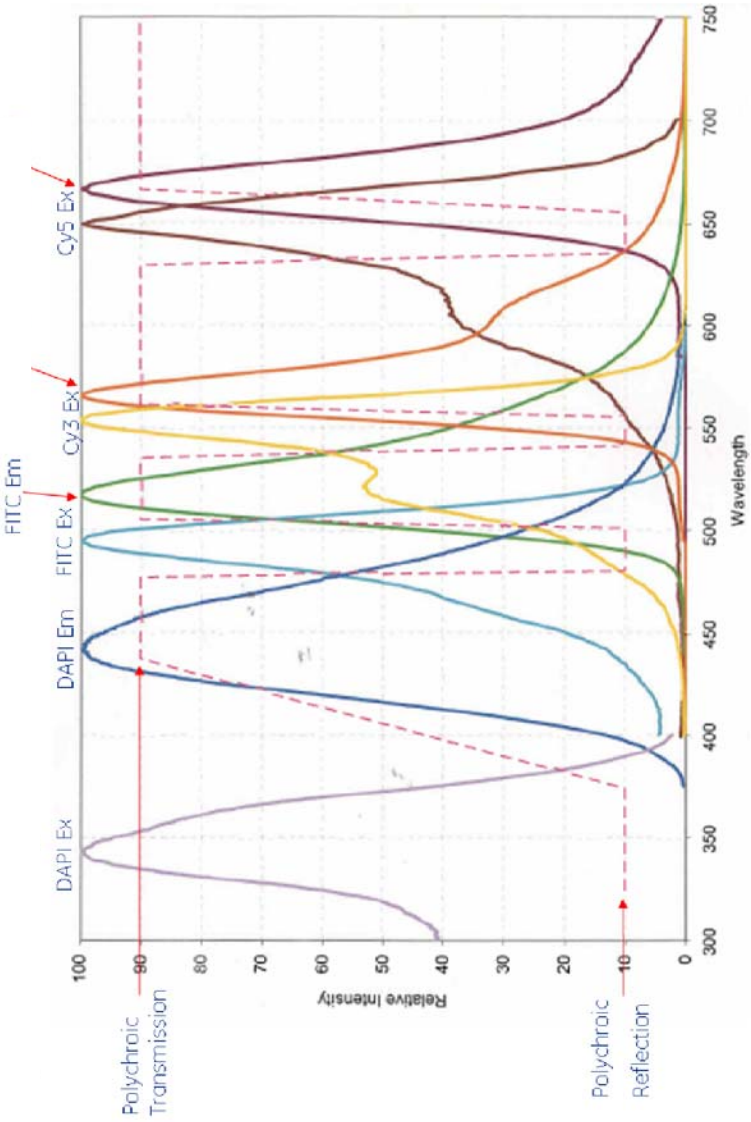


Fig 10-11. QUAD 2 Polychroic [X]

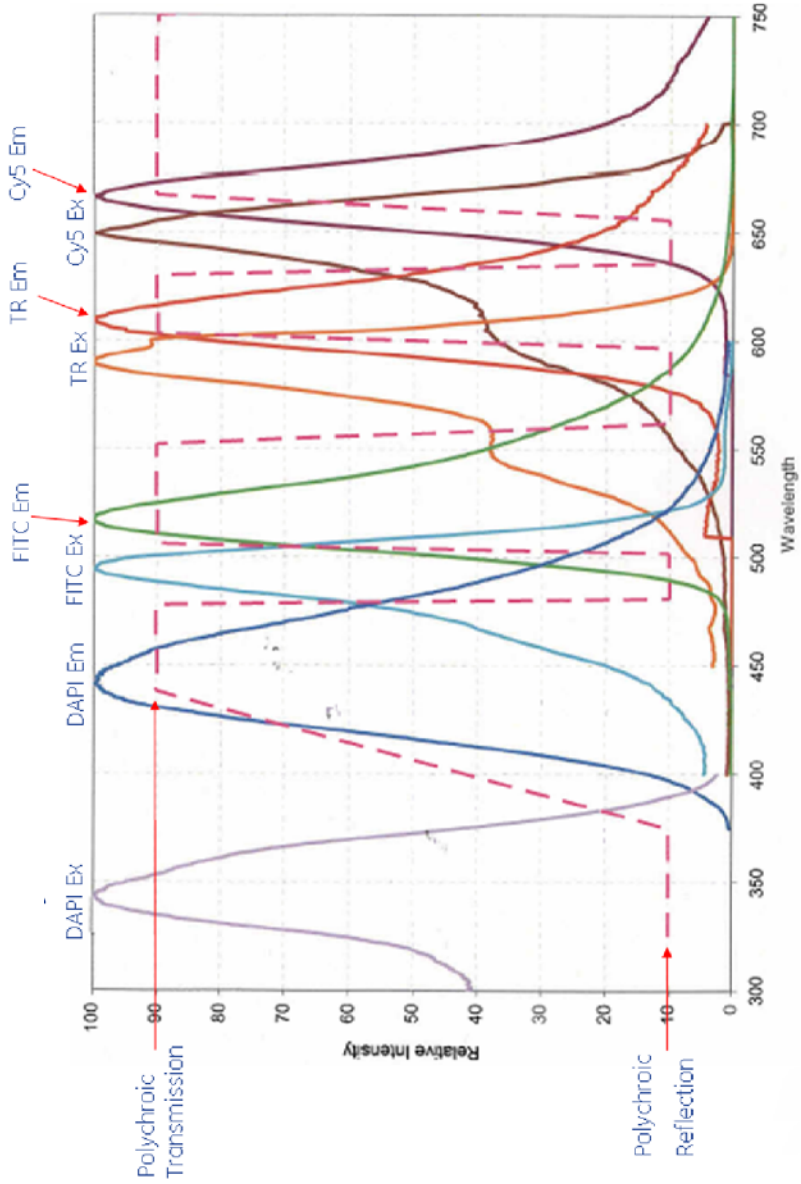


Fig 10-12. QUAD 3 Polychroic [Z]

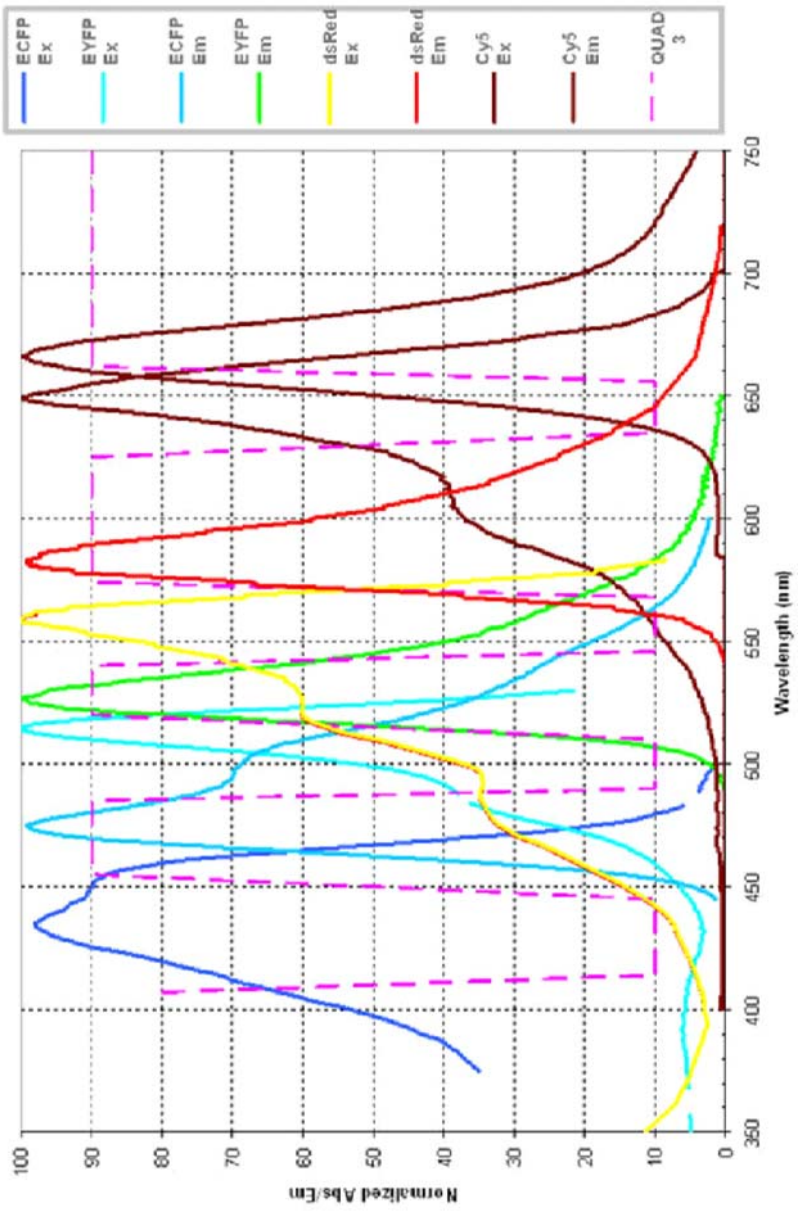
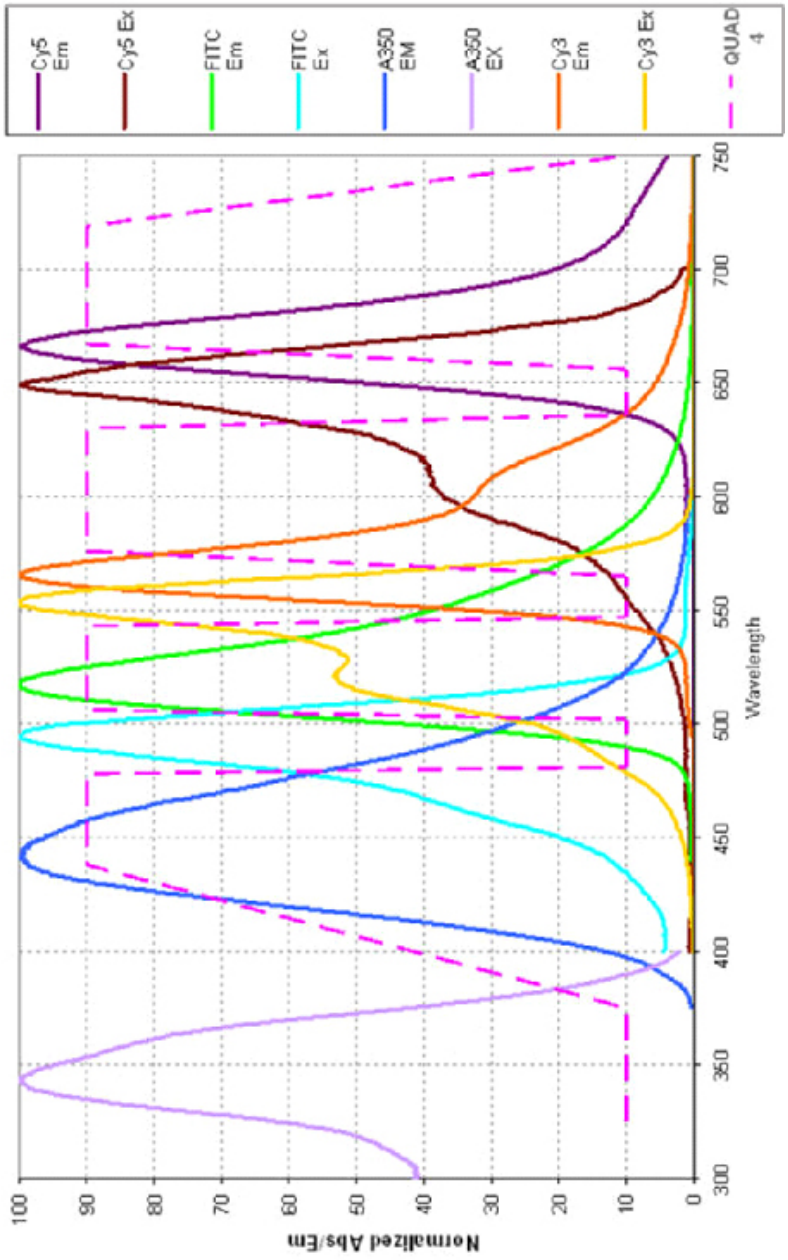


Fig 10-13. QUAD 4 Polychroic [A]



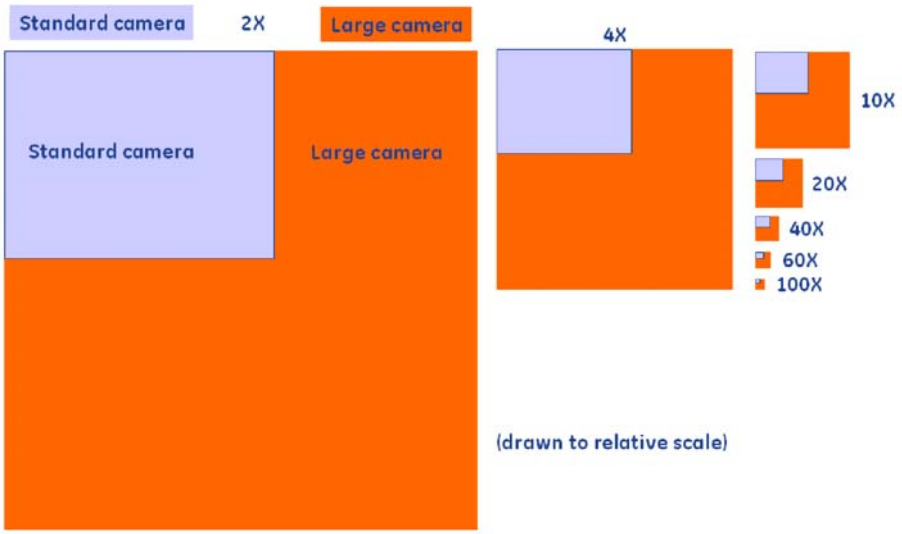
A.2 Camera Options

Large Chip CCD Camera
CoolSNAP K4
2048 x 2048 pixel array
7.40µm square pixel

Standard Chip CCD Camera
CoolSNAP ES2
1392 x 1040 pixel array
6.45µm square pixel

Objective Mag / NA	Large camera 2048 x 2048 7.4 µm / pixel		Standard camera 1392 x 1040 6.45 µm / pixel	
	Pixel size (µm)	FOV x-y (mm)	Pixel size (µm)	FOV x-y (mm)
2X / 0.1	3.70	7.58x7.58	3.23	4.49x3.35
4X / 0.2	1.85	3.79x3.79	1.61	2.25x1.68
10X / 0.45	0.74	1.52x1.52	0.65	0.90x0.67
20X / 0.45	0.37	0.76x0.76	0.32	0.45x0.34
20X / 0.75	0.37	0.76x0.76	0.32	0.45x0.34
40X / 0.60	0.19	0.36x0.36	0.16	0.22x0.17
40X / 0.95	0.19	0.36x0.36	0.16	0.22x0.17
60X / 0.70	0.12	0.25x0.25	0.11	0.15x0.11
60X / 0.95	0.12	0.25x0.25	0.11	0.15x0.11
100X / 0.90	0.07	0.15x0.15	0.06	0.09x0.07

A.3 Field of View (Standard vs Large camera)



A.4 Theoretical Depth of Field Measurements

Theoretically predicted imaging characteristics of IN Cell Analyzer 2000

	Wavelength	550	nm
Standard Camera	CCD pixel	6.45	um
	no. of pixels, X	1392	
	no. of pixels, Y	1040	
Large Camera	CCD pixel	7.4	um
	no. of pixels, X	2048	
	no. of pixels, Y	2048	

IN Cell 2000 imaging characteristics with its standard camera

Dry Objective	Mag	NA	WD, mm	Corr Collar	Image pix size, um	FOV, X*Y mm * mm	Lateral Res, um	Depth of Field, um
2X / 0.1	2	0.10	8.50	no	3.23	4.489 * 3.354	6.45	87.25
4X / 0.2	4	0.20	20.00	no	1.61	2.245 * 1.677	3.23	17.78
10X / 0.45	10	0.45	4.00	no	0.645	0.898 * 0.671	1.29	3.00
20X / 0.45	20	0.45	7.50	0.0-2.0	0.323	0.449 * 0.335	0.75	2.80
20X / 0.75	20	0.75	1.00	no	0.323	0.449 * 0.335	0.65	1.02
40X / 0.6	40	0.60	3.20	0.0-2.0	0.161	0.224 * 0.168	0.56	1.55
40X / 0.95	40	0.95	0.15	0.11-0.23	0.161	0.224 * 0.168	0.35	0.62
60X / 0.7	60	0.70	1.80	0.1-1.3	0.108	0.15 * 0.112	0.48	1.13
60X / 0.95	60	0.95	0.15	0.11-0.23	0.108	0.15 * 0.112	0.35	0.62
100X / 0.9	100	0.90	0.3	0.14-0.20	0.0645	0.09 * 0.067	0.37	0.68

IN Cell 2000 imaging characteristics with its large camera

Dry Objective	Mag	NA	WD, mm	Corr Collar	Image pix size, um	FOV, X*Y mm * mm	Lateral Res, um	Depth of Field, um
2X / 0.1	2	0.10	8.50	no	3.70	7.578 * 7.578	7.40	92.00
4X / 0.2	4	0.20	20.00	no	1.85	3.789 * 3.789	3.70	18.38
10X / 0.45	10	0.45	4.00	no	0.740	1.516 * 1.516	1.48	3.04
20X / 0.45	20	0.45	7.50	0.0-2.0	0.370	0.758 * 0.758	0.75	2.80
20X / 0.75	20	0.75	1.00	no	0.370	0.758 * 0.758	0.74	1.03
40X / 0.6	40	0.60	3.20	0.0-2.0	0.185	0.379 * 0.379	0.56	1.55
40X / 0.95	40	0.95	0.15	0.11-0.23	0.185	0.379 * 0.379	0.37	0.62
60X / 0.7	60	0.70	1.80	0.1-1.5	0.123	0.253 * 0.253	0.48	1.13
60X / 0.95	60	0.95	0.15	0.11-0.23	0.123	0.253 * 0.253	0.35	0.62
100X / 0.9	100	0.90	0.3	0.14-0.20	0.074	0.152 * 0.152	0.37	0.68

Lateral resolution (Rxy) is calculated as the larger of the two: Rayleigh's definition, Rxy = 0.61 /(NA), or 2*(size of image pixel) [based on Nyquist's sampling theorem].

Notice to purchaser
The IN Cell Analyzer 2000/2200 system is for research purposes only. It is not approved for diagnosis of disease in humans or animals.

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