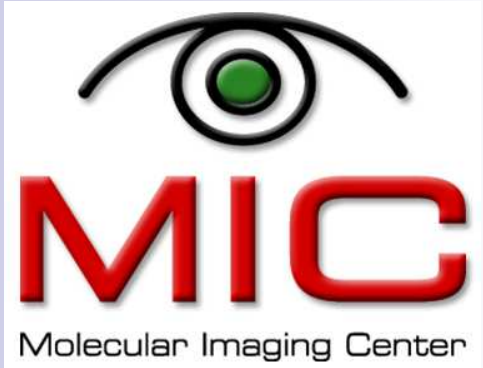
BD Pathway 855

Quick user guide to the Automatic fluorescent microscope/High-Throughput Screener (HTS)



Lasse Evensen (March 2012)

Starting up the instrument and imaging software

- Insert the desired objective
- Turn on the main switch
- Turn on the computer located in the cabinet
- Wait for the green ”Ready” lamp on the front of the microscope to light up (if it doesn’t, re-start the microscope). The ”Ready” lamp assures that all movable parts are in the correct starting position
- When the “Ready” lamp is lit, turn on the mercury lamp(s) (Lamp A is for GFP, Hoechst and PI. Lamp B is for Tritc, Alexa 647 and Alexa 546)
- Type in the password to access the computer
- Open Attovision software and choose your username (no password required)
- Never turn the lamps off and directly on again! If the lamps are turned off you must wait 30 minutes before turning them on again

Introduction to the software

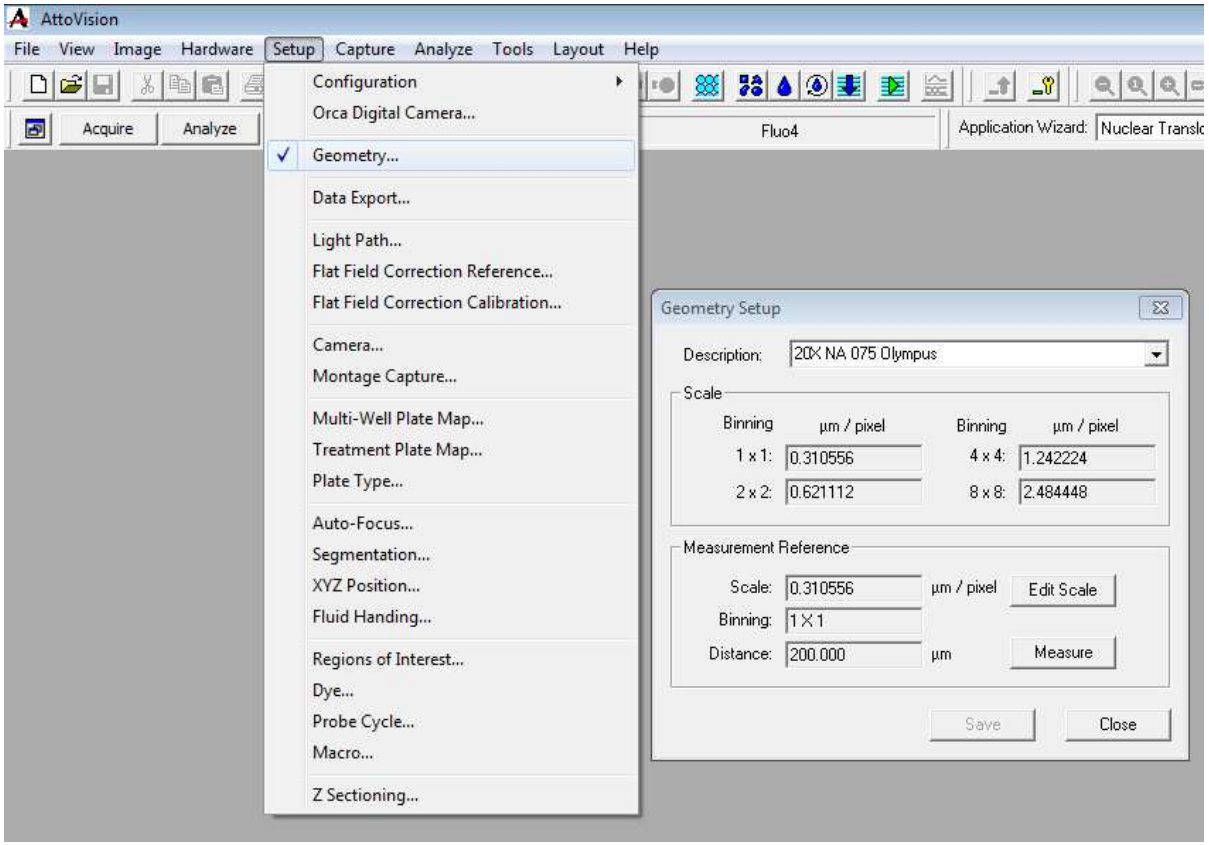
BD Pathway 855 BioImager

To perform image acquisition:

Some general info……

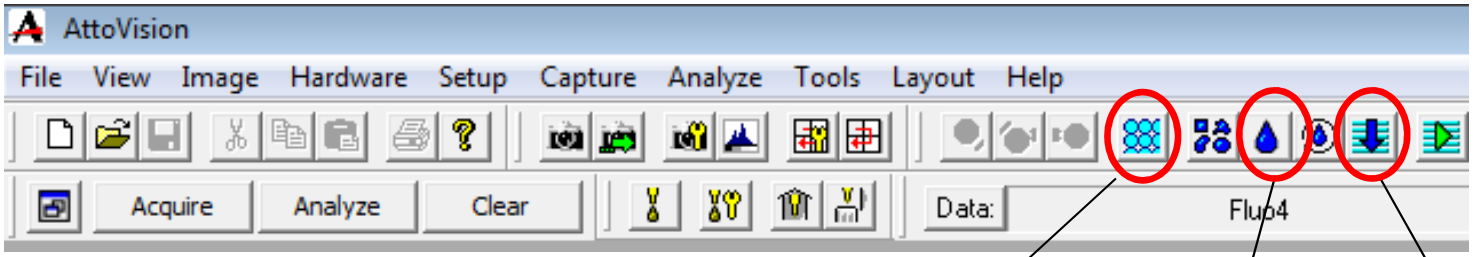
- The macro is the process that ”tells” the Pathway what to do
- It consists of four main steps: (just as manual microscopy)
  - Positioning of the objective (on the pathway the plate is stationary and the objective move around underneath)
  - Finding the focal plane
  - Chose of filters
  - Image acquisition
- When performing multicolor fluorescence imaging, filter combinations must be defined and will be demonstrated later (Slide 15)
- If the microscope does not perform as desired there is most likely something wrong with the macro!

First, define which objective you inserted!



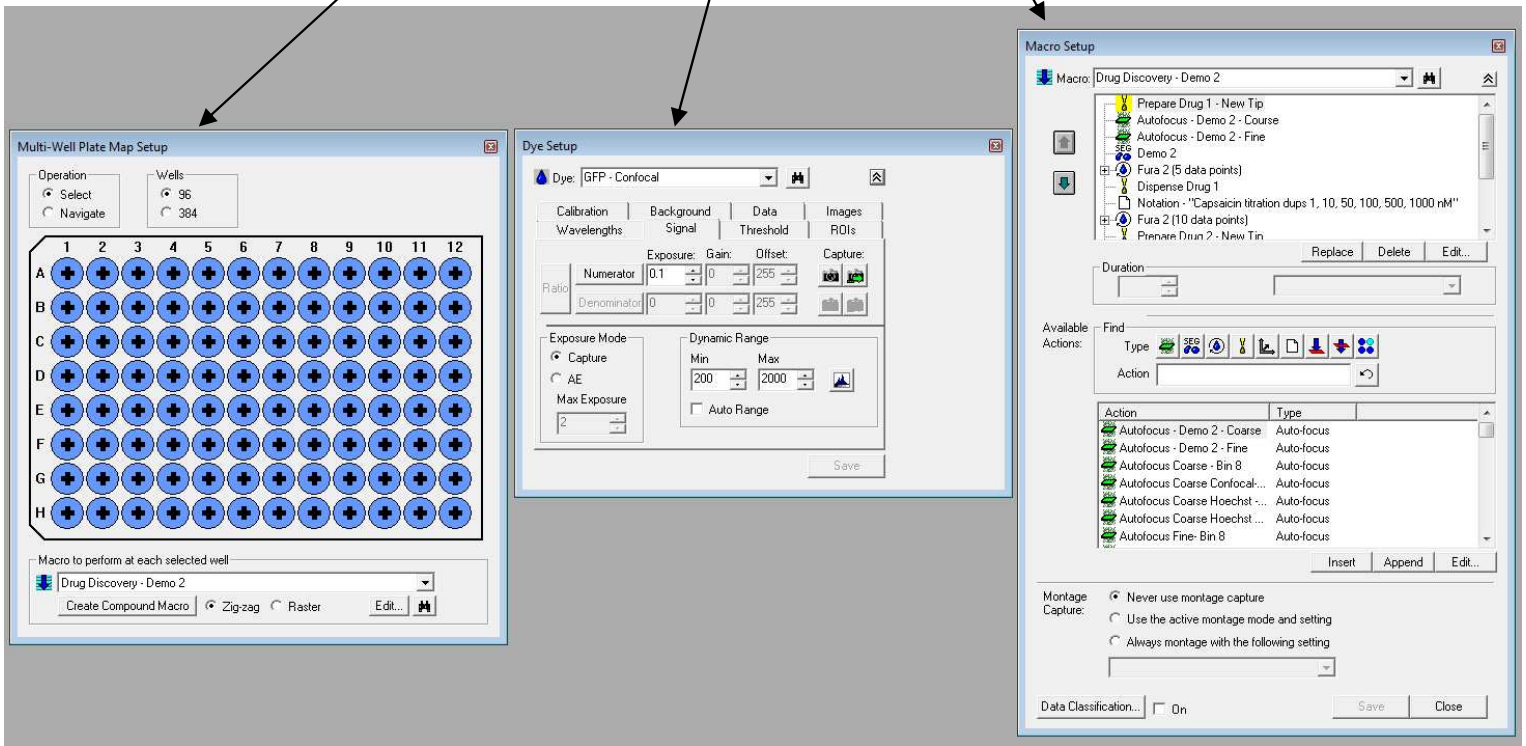
1. Click on ”Setup” on the main menu at the top
2. Click on ”Geometry”
3. From the ”Description” drop-down menu in the “Geometry Setup” window, set the correct objective

The Attovision software: Three important buttons



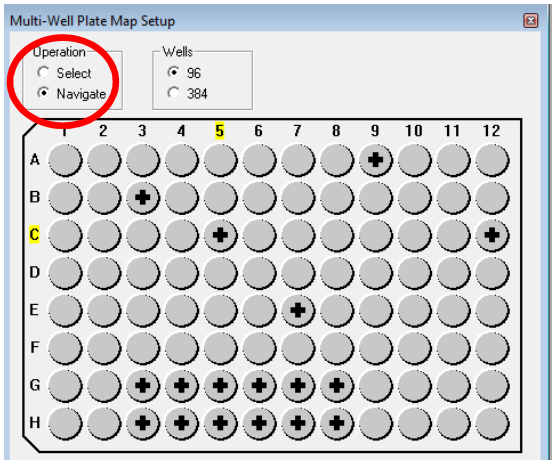
These three buttons <u>controls (from the left):</u>

1. Navigation of the objective, which wells are going to be imaged
2. <u>Dye Setup:</u> Filter control (chose your filter, exposure time and gain)
3. <u>Macro setup:</u> The window where you program the microscope

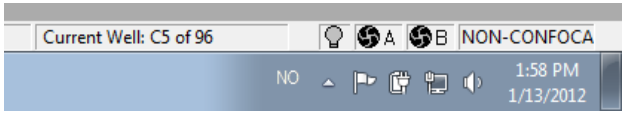


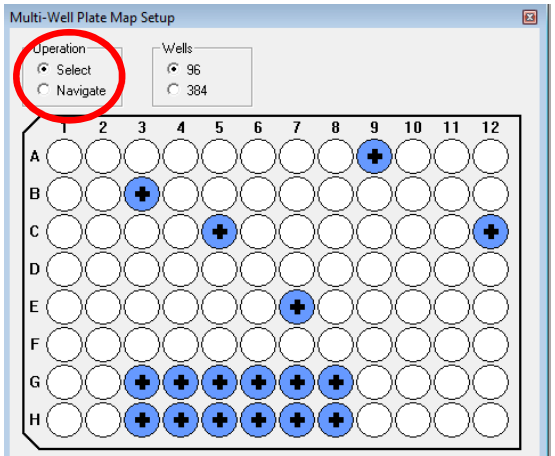
The Plate Map Setup Window

Controls what plate type you have (96- or 384-well plate) and which wells you want to image. In manual mode it controls which well you want to navigate the objective to.



In the navigate mode (red circle), if you click on a well the objective will locate to that well. For example, click on C5 and the objective will locate under C5. On your screen down to the left, the software will inform you about the location of the objective





In the select mode (red circle), if you click on a well it will appear blue with a plus sign in the middle. That means that you later want to include that well in the automatic imaging. You can select single wells or the whole plate

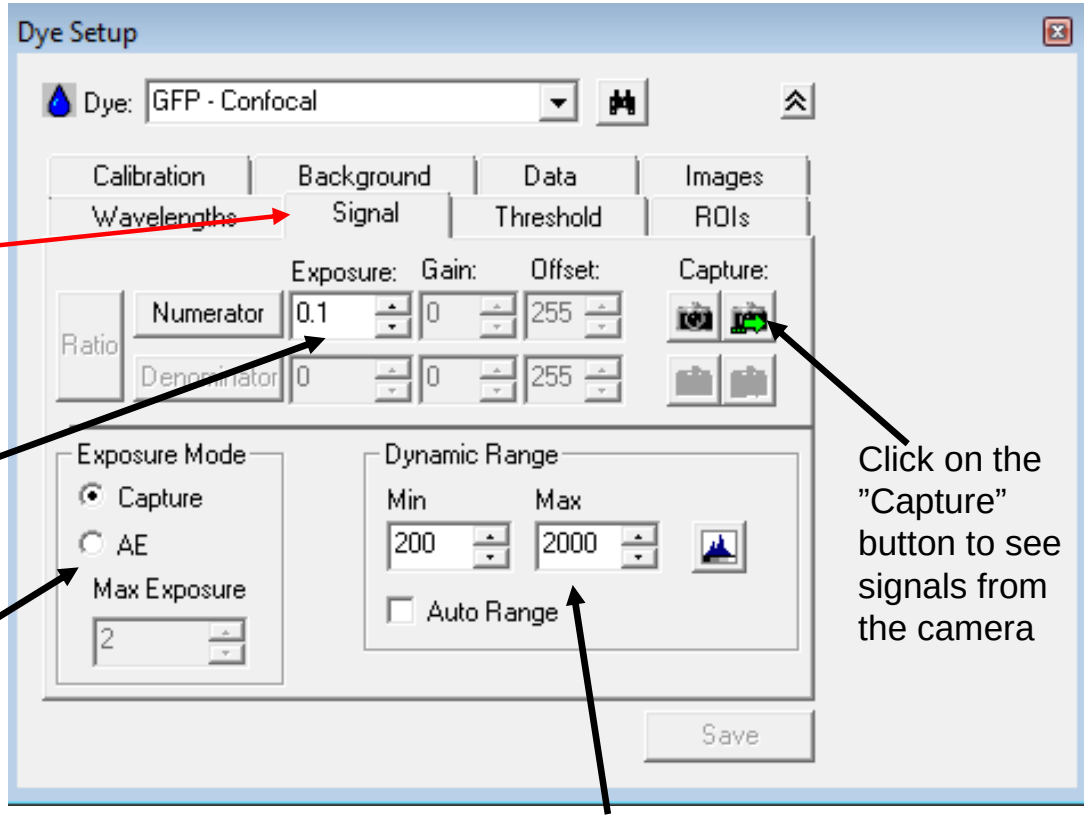
The Dye Setup Window (signal tab)

Chose dye from the drop-down menu

Be sure you are on the signal tab

Adjust exposure time (seconds) by typing in a number or click on the arrows. The same for Gain, choose a number between 0 and 255.

Autoexposure (AE) sets the exposure time automatically



Click on the "Capture" button to see signals from the camera

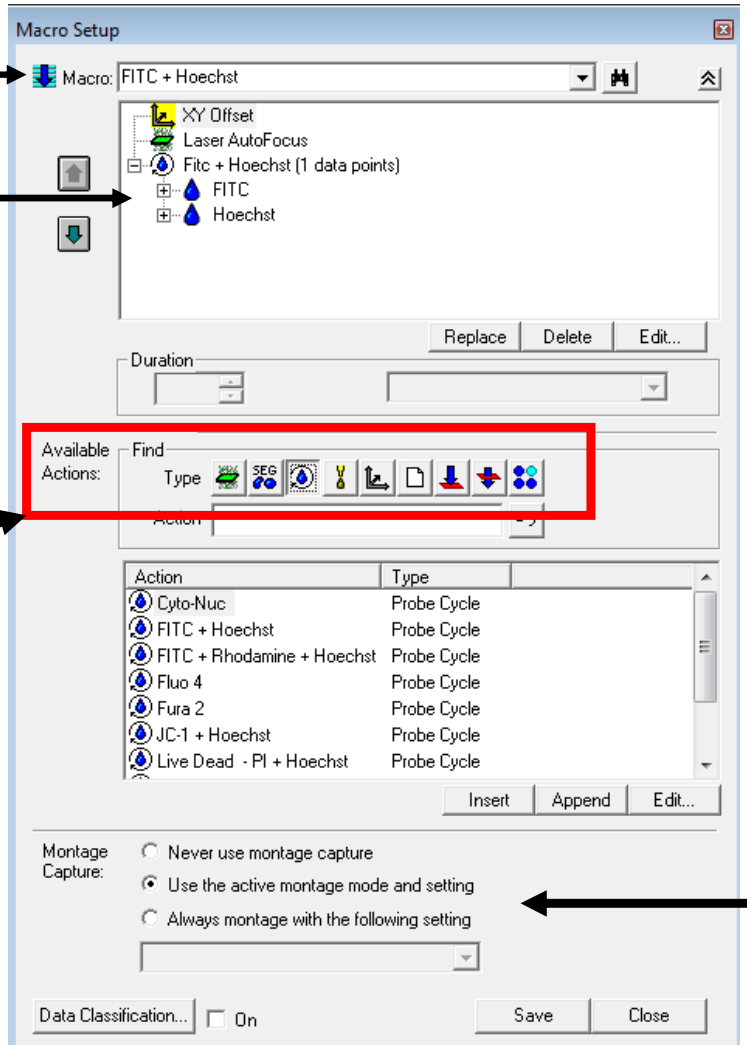
Dynamic range let you control the distribution of pixel intensity. Extremes are 0 as Min and 4095 as Max (widest distribution). Min:200 and Max:2000 means that pixel intensity is distributed in a smaller range giving you a more intense image. Auto Range sets the dynamic range automatically

The Macro Setup Window

From the drop-down menu, find your macro

The steps in the macro performed in each well selected for imaging

(XY Offset: Positioning of the object, Laser Autofocus: Finding the focus plane, Fitc+Hoechst; the selected dye (filter) combination



Available actions:

Click on each icons to get a list of available focusing methods, filter combinations, objective positions etc. The list will appear in the window below. As an example, available filter combinations are shown

Montage capture control (stiching of tiles to image a larger viewfield): If montage capture is desired it is chosen here

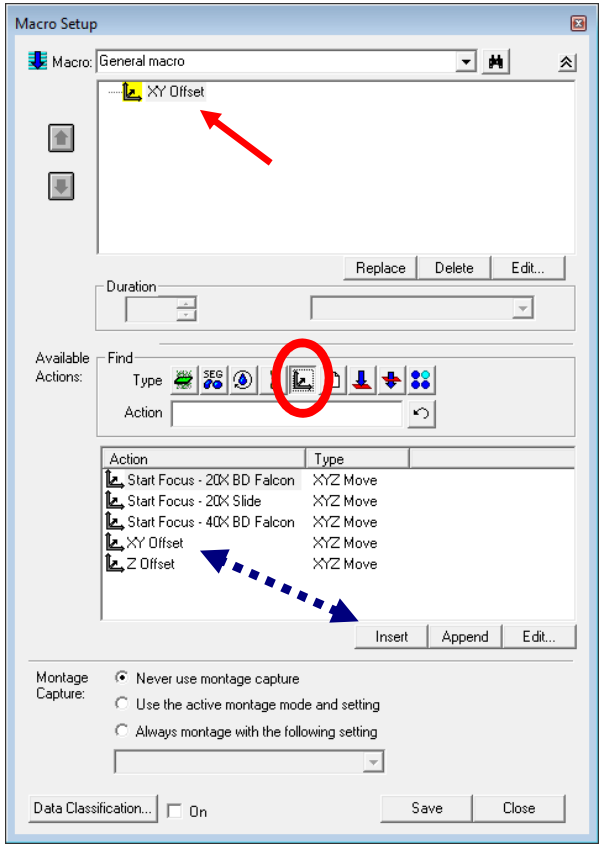
Building a macro

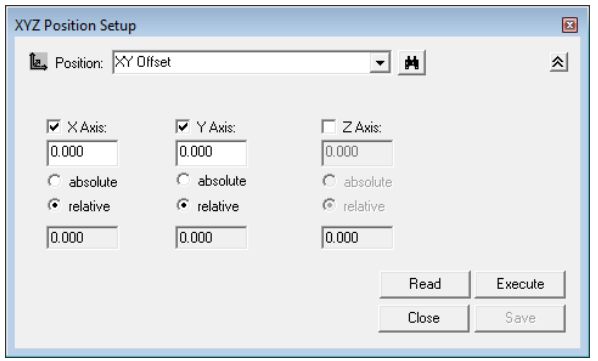
(How to instruct the machine
to behave as you want)

Step by step protocol

Program the microscope: Macro setup – **Object position**

- Open the macro setup window
- Click on the XYZ-position icon (red circle)
- Click on XY-offset and click on the ”Insert” button (dotted blue arrow)
- Double click on the XY-offset command line (red arrow) and the ”xyz-position setup”window opens. Here you can choose the coordinates in the well the objective shall travel to during imaging
- If zero is typed in for x- and y-axis the objective will travel to the middle of the well

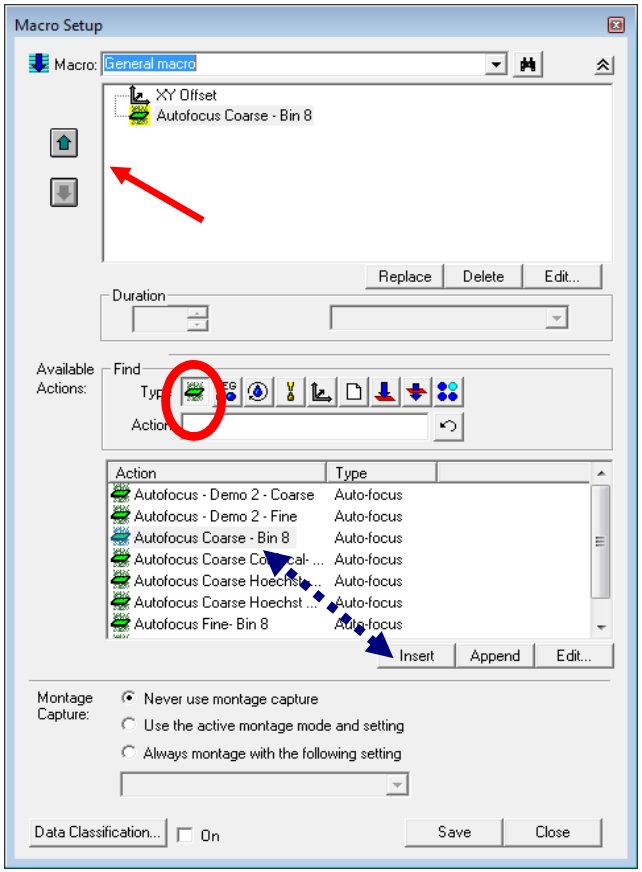




Program the microscope:

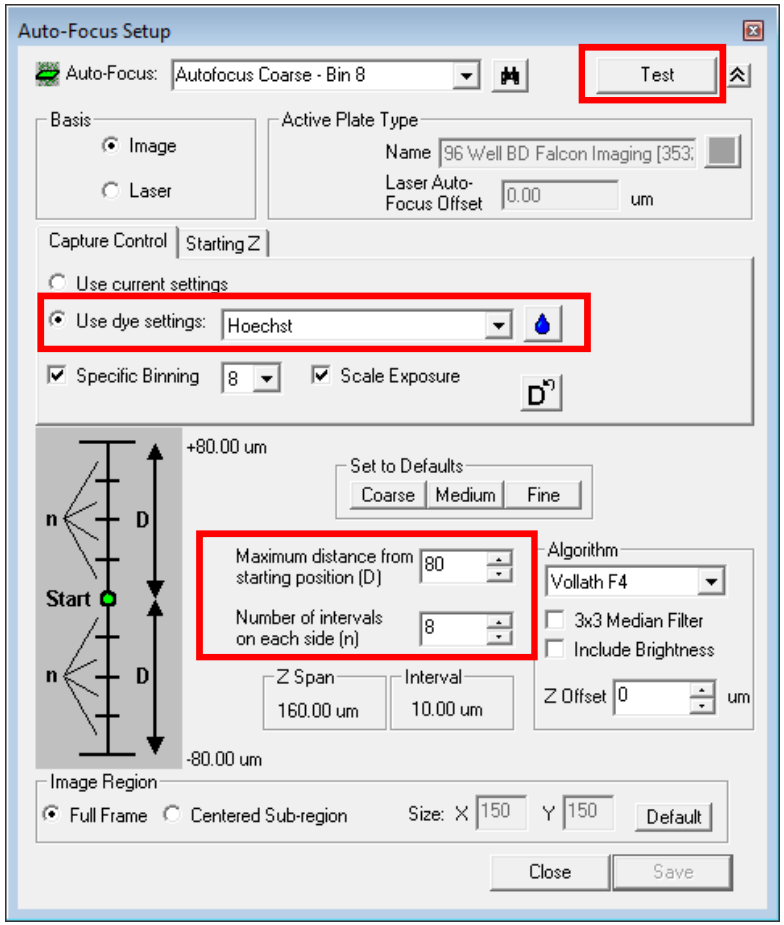
Macro setup - **Autofocus**

- Click on the Autofocus action button (red circle)
- Click on Autofocus Coarse – Bin 8 and click insert (blue dotted line)
- Use the arrow buttons on the top left (red arrow) to set the order of the actions. XY-offset should be first followed by Autofocus
- Double click on the Autofocus Coarse – Bin 8 command line and the “Autofocus Setup” command window will appear (Next slide)



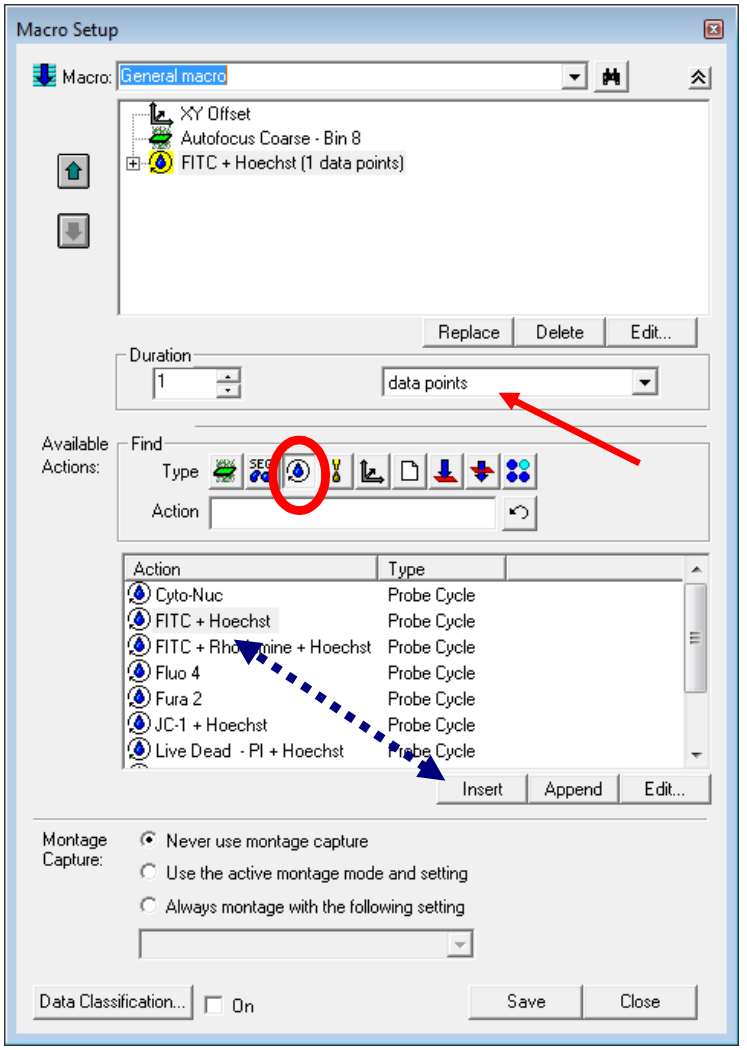
Program the microscope: Macro setup - **Autofocus**

- Check the box called ”Use dye settings” and choose which filter you want to use to find the focal plane.
- Hint: If cells are stained with Hoechst this is a nice dye to find the focus
- The cartoon explains how the autofocus work:
  - In the “dye setup” control window (slide 8) click the capture button and use the focusing wheel to find the focus. If cells are stained with Hoechst use this filter
  - During autofocus the objective starts from the z-posistion you left it in. Based on the number you type in in the box ”Maximum distance from starting position” (D) the objective travels up the indicated distance D. In this example it travels 80 µM upwards.
  - In the box ”Number of intervals on each side” (n) you choose how many times an image shall be acquired during distance D. In this example n=8 meaning that 8 images are acquired as the objective travels distance D.
  - The operation is repeated below the starting position. The objective travels 80 µM below the starting position and acquires 8 images.
  - In total, after autofocus is performed 16 images are acquired and the computer software uses an algorithm to choose from these images and picks the one where the cells are in focus.
  - The chosen image sets the focal plane for acquisition of images in that well
  - When adjusting autofocus in order to bring the cells in focus nicely: Adjust n and D and click the “test”-button until the machine is able to bring the cells into focus automatically
  - Test the focus-settings for the wells furthest away from each other. If focus is found in these two wells it is most likely that it will be found for all the wells in between.



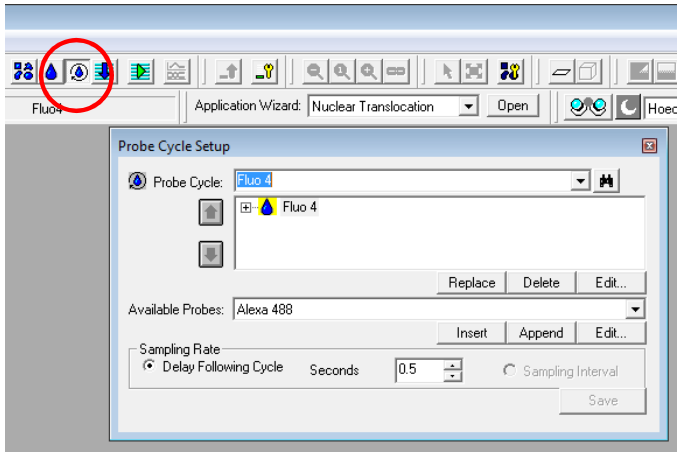
Program the microscope:
Macro setup – **Choice of filters**

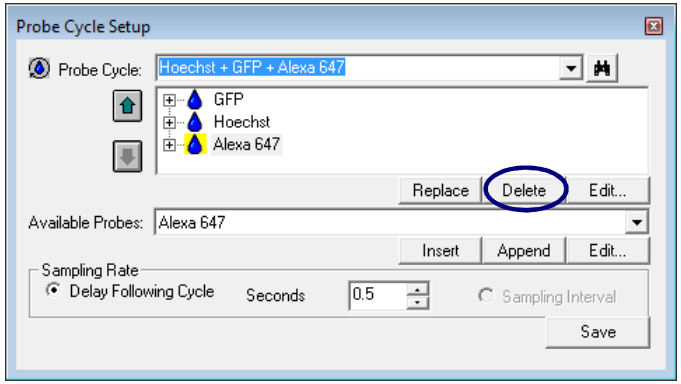
- Click on the dye setup button (red circle)
- Click on FITC+Hoechst and click insert (to make new filter combinations see next slide)
- Use the arrows on the top left to put the imaging action after autofocus
- Make sure that it is indicated that you want to acquire ”1 datapoints” (red arrow)

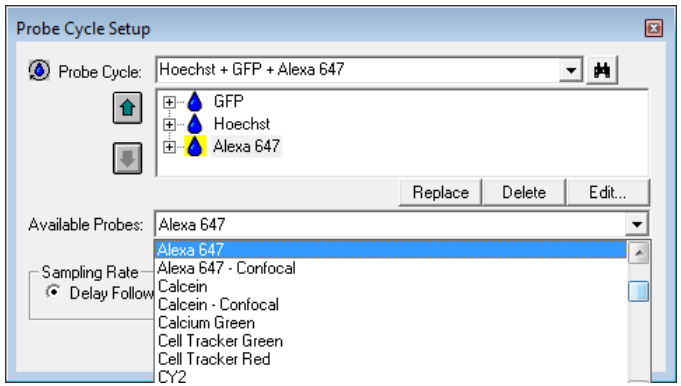


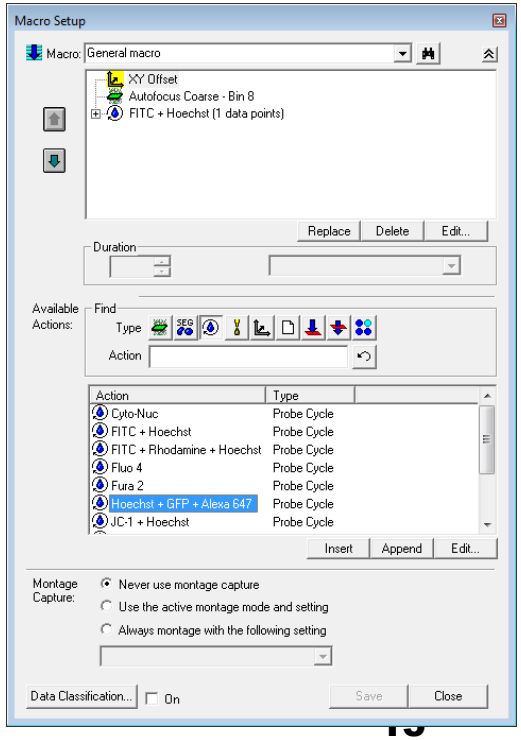
How to set up new filter combinations (Probe Cycle)

- Click on the symbol in the red circle
- In the Probe Cycle Setup window: Give the filter cobination a name
- Delete any filters that were listed from before (blue circle)
- From the drop-down menu in ”Probe Cycle Setup” window, find the filters you want to include in the probe cycle.
- Click ”Insert” button
- Arrange the order of the filters by using the green arrows on the left in the ”Probe Cycle Setup” window. Hint: If you want to use Hoechst as filter to find focus, Hoechst should be the first filter in the probe cycle
- By clicking the ”Macro Setup” button (slide 6) the ”Macro Setup” window will appear. The new filter combination is now available for use in your macro.



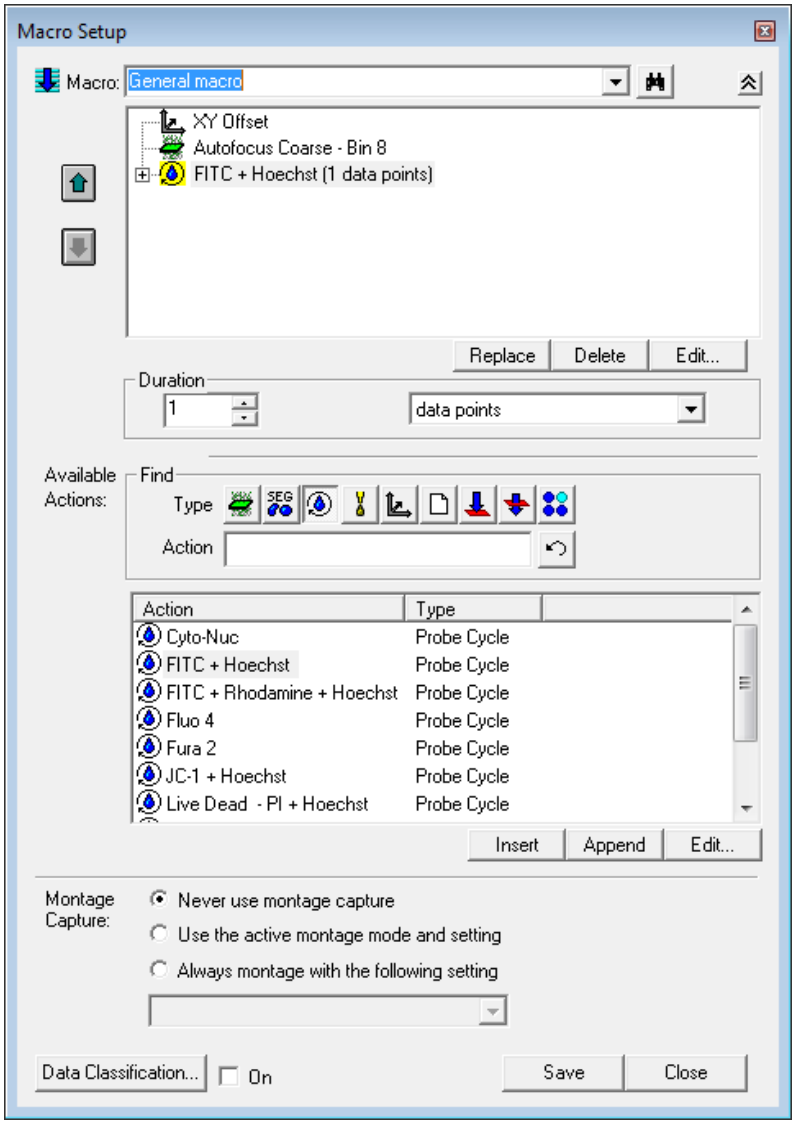




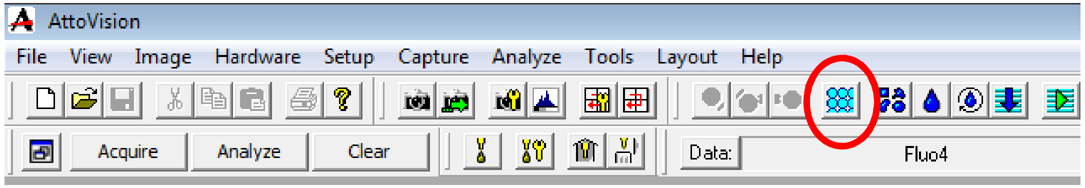


The Macro

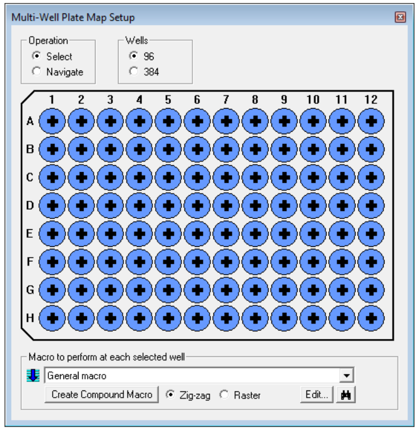
- The macro is now complete. The position of the objective is programmed, the autofocus step brings the cells into focus and since cells express GFP and are stained with Hoechst the filter combination FITC+Hoechst is chosen
- The macro is only generated once for each imaging task. Once you have made a macro you use it over and over again. You only need to check the autofocus everytime you use the imager.
- The macro is a program instructing the microscope of what to do in each well you want to image
- What you have not told the microscope yet is which wells that are going to be imaged
- See next slide for instructions of how to import well locations into your macro

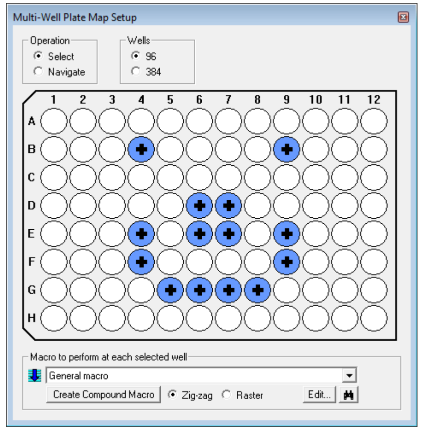


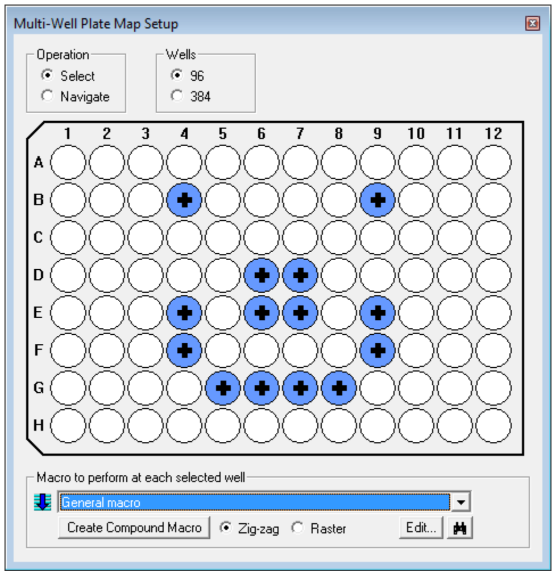
How to import well locations into your macro

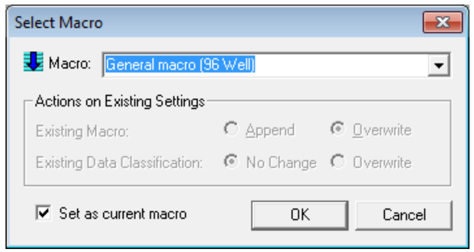


- Open the ”multi-well plate map setup window” (red circle)
- To choose all the wells on a plate: In the select mode click and drag the mouse pointer along the plate while you holde down the left button on the mouse.
- To choose single wells: Click on the wells you want to include in your macro
- From the drop-down menu at the bottom of the window find the name of your macro and click ”create compound macro”
- The ”Select Macro” window will appear and a new macro with the extension “96-well” will be generated. This is how you prevent to overwrite the “mother macro”. You can always go back to the “mother macro” and import well locations, hence, you do not need to build a new macro every time you are going to do imaging
- Click “OK” in the “Select Macro” window and your well location will be imported into the macro



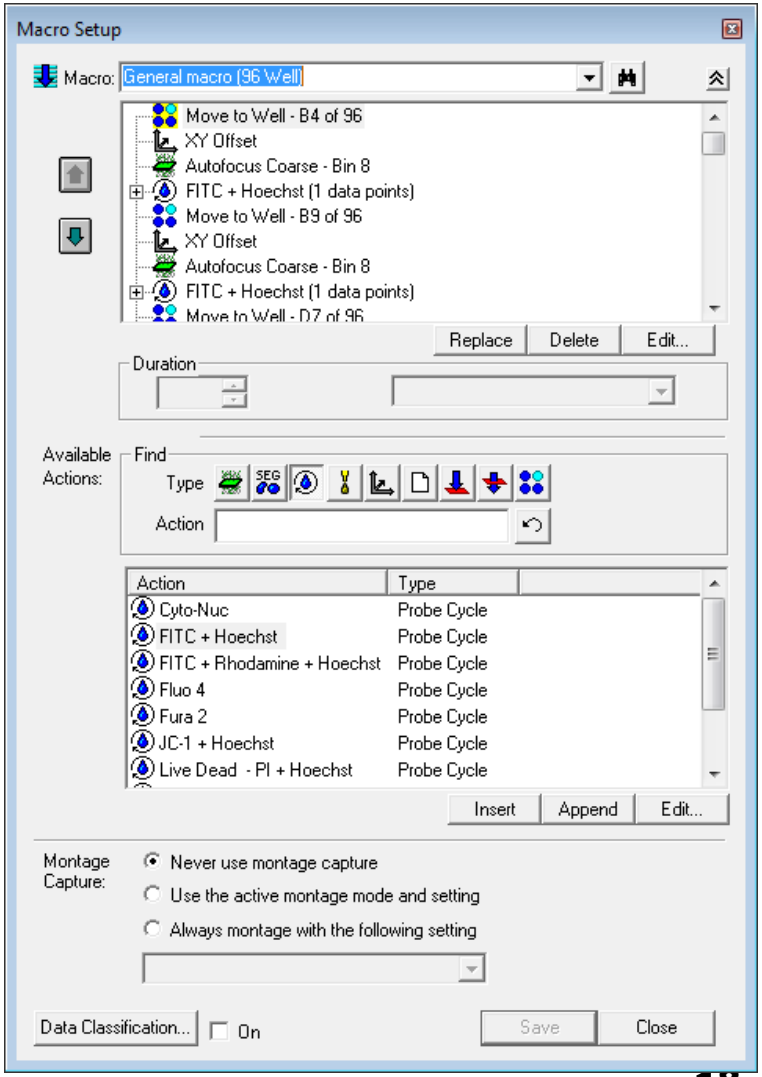




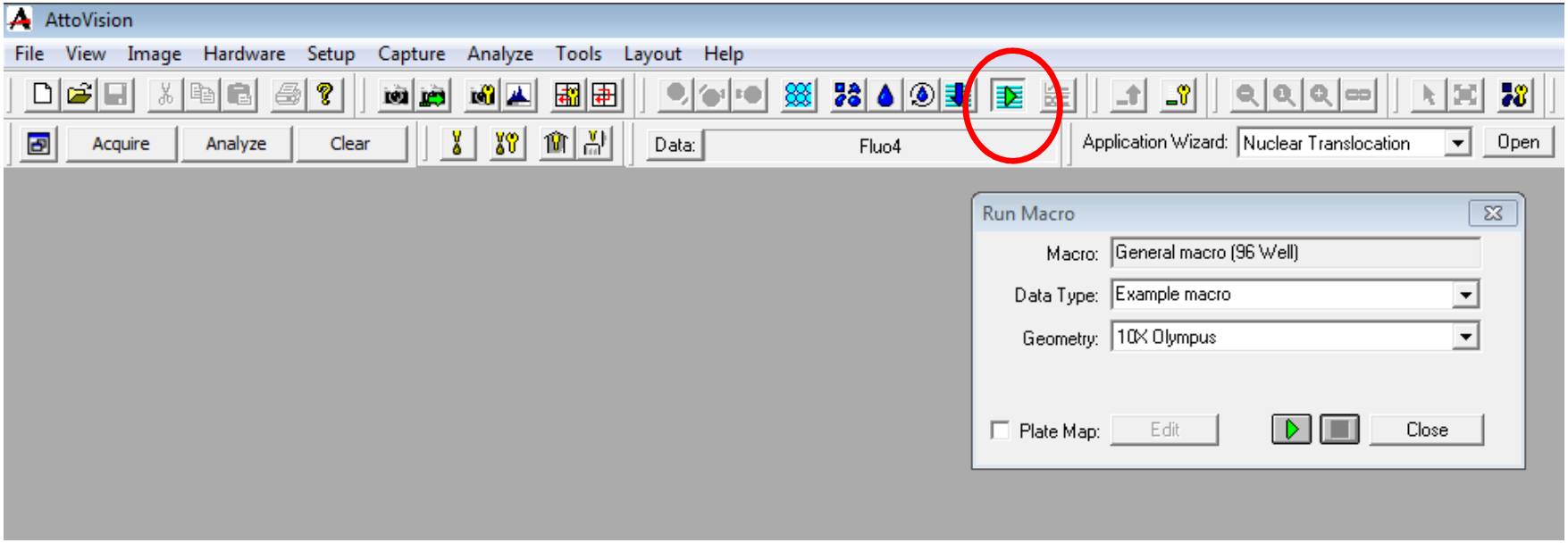


Steps in the macro

- The objective will now travel to well B4
- It will move within B4 to the coordinates you typed in.
- It will use the autofocus settings and find the focal plane of the cells
- Finally, it will acquire images using the FITC and Hoechst filters
- Then it will move to well B9 and repeat the process there
- It will repeat the process for all the wells you have programmed the microscope to image
- In this example, separate images for FITC and Hoechst will be saved and they can later be quantified separately



How to start imaging



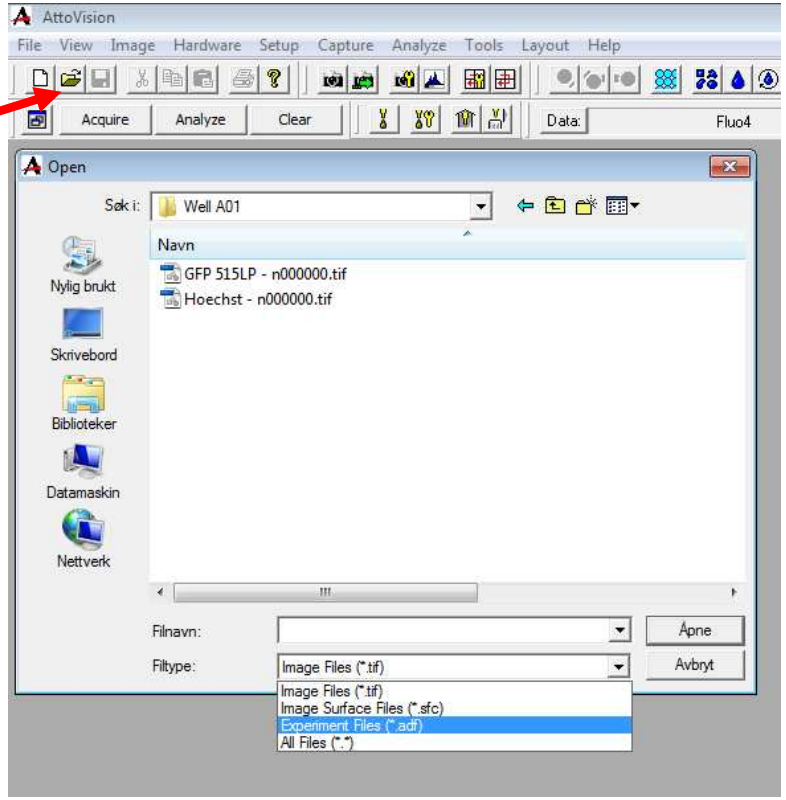
- Click on the ”Play” button (red circle)
- The ”Run Macro” window will appear. Give your experiment a name (Data type). The data will be saved in a folder with the same name as you type in the ”Data type” box.
- If not already done, choose which objective you are using
- Click ”Play” symbol in the ”Run Macro” window
- Imaging will start after about 30 seconds

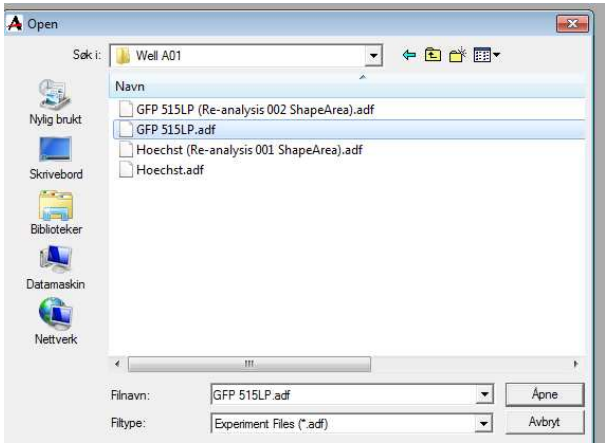
Export of images

How to obtain pseudocolours and merge different channels to one image

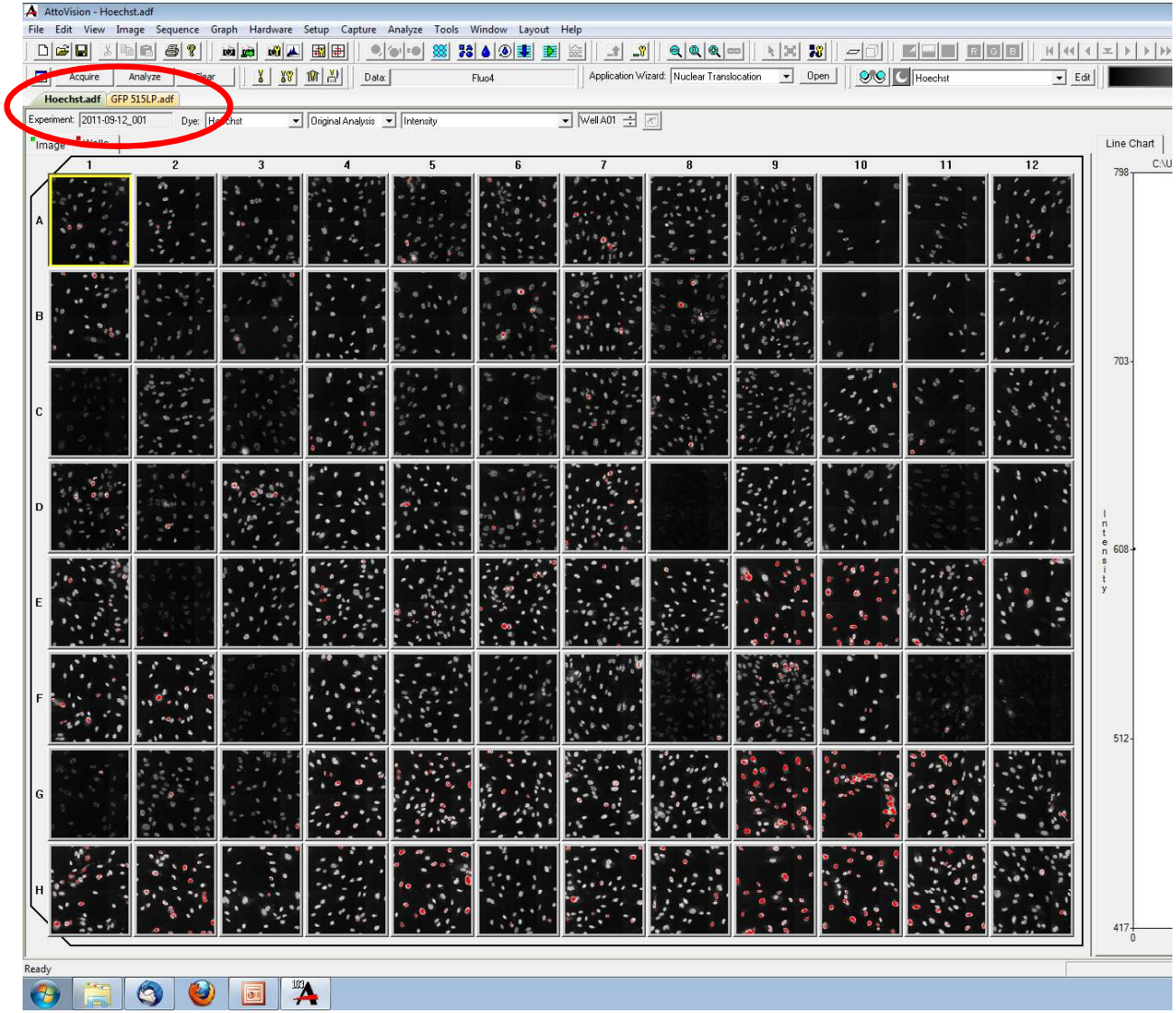
Open the experiment containing the images of interest

1. Click on the open folder icon and locate your experiment
2. In the experiment folder open a random well folder
3. In the random well folder choose *.adf as filetype (see upper image)
4. Open the channels you want to merge. In this example the channels are GFP515LP and Hoechst (lower image). Choose ”GFP515LP.adf” and click open
5. To open more than one channel repeat step 3 and 4. In this example, to open Hoechst images in addition to the GFP515 images choose ”Hoechst.adf” and click open





After you opened the channels you want to merge this screen will appear. The images are thumbnails and gives you an overview of the plate imaged. You can get the overview for the different channels by clicking on the tabs containing the name of the dyes (see red circle)

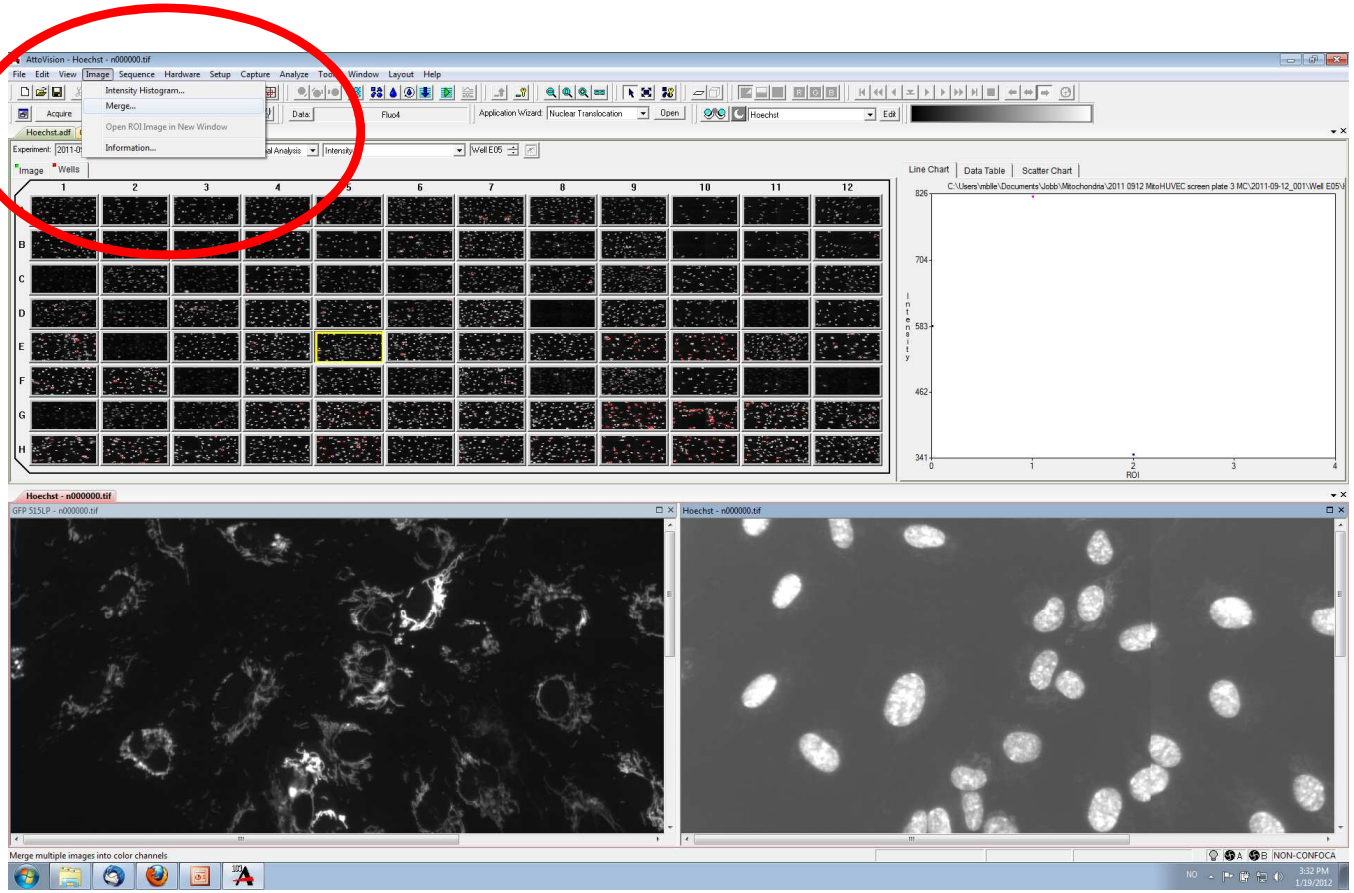


Next, to open all the images acquired for one single well do the following:

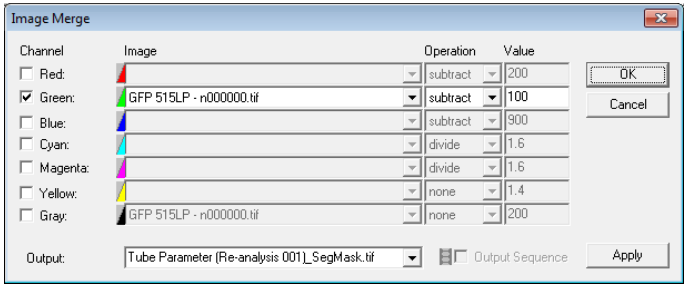
Hold down *shift+ctrl* and click on the well you want to export images from.

For example: hold down *shift+ctrl* and click on well E5. In this example two images are acquired in this well and they will appear on the screen (see next slide).

How to merge two channels into one image



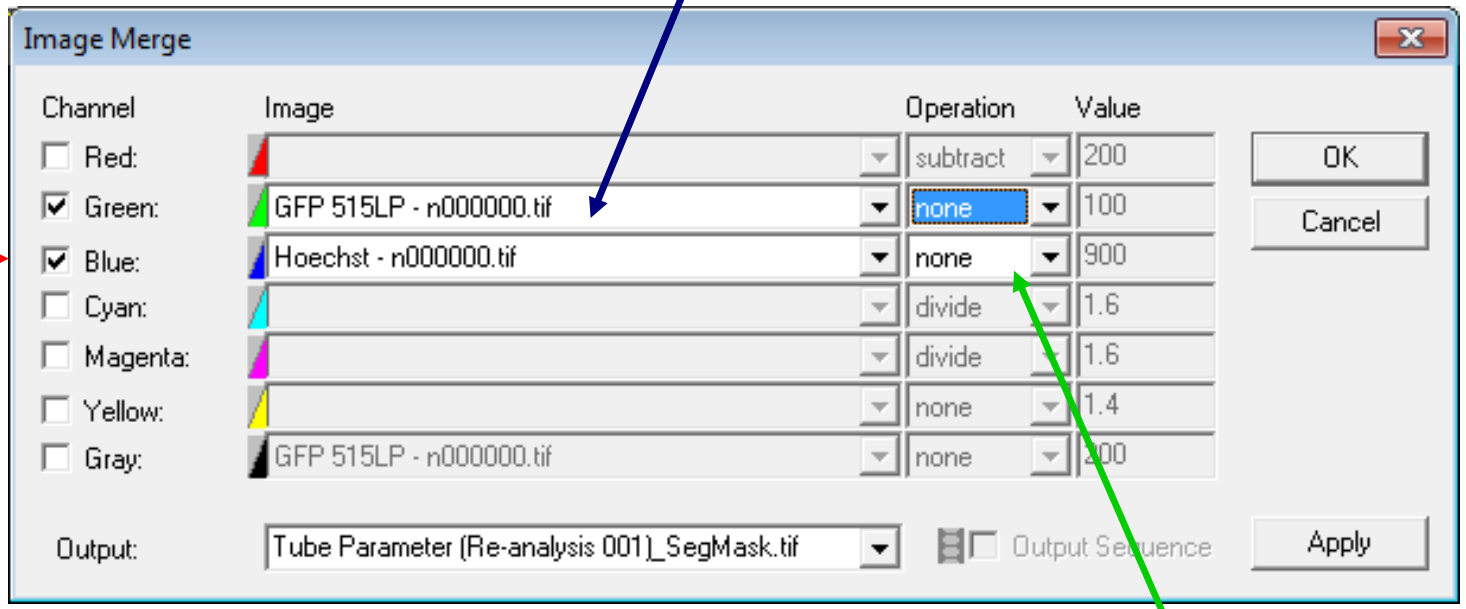
As indicated with the red circle, click on the ”Image” tab at the main menu and choose ”Merge”. The window beneath will appear:



Merging two channels

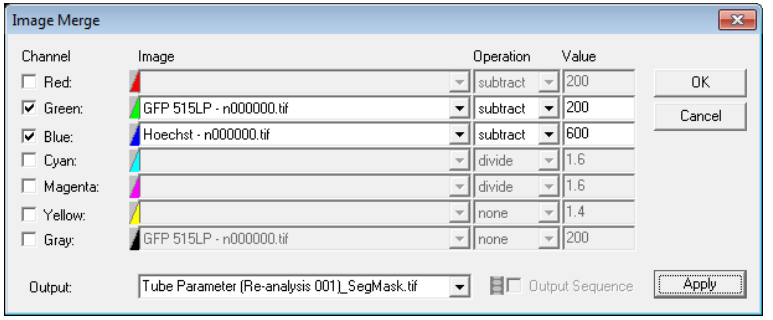
Check the boxes for the colours you want on your image (pseudocolors). In this example the cells are stained with Hoechst and express GFP. Therefore, check the boxes for ”Blue” and ”Green”

Choose which image that are going to the two channels. In this example GFP515LP is put in the green channel and Hoechst is put in the blue channel.

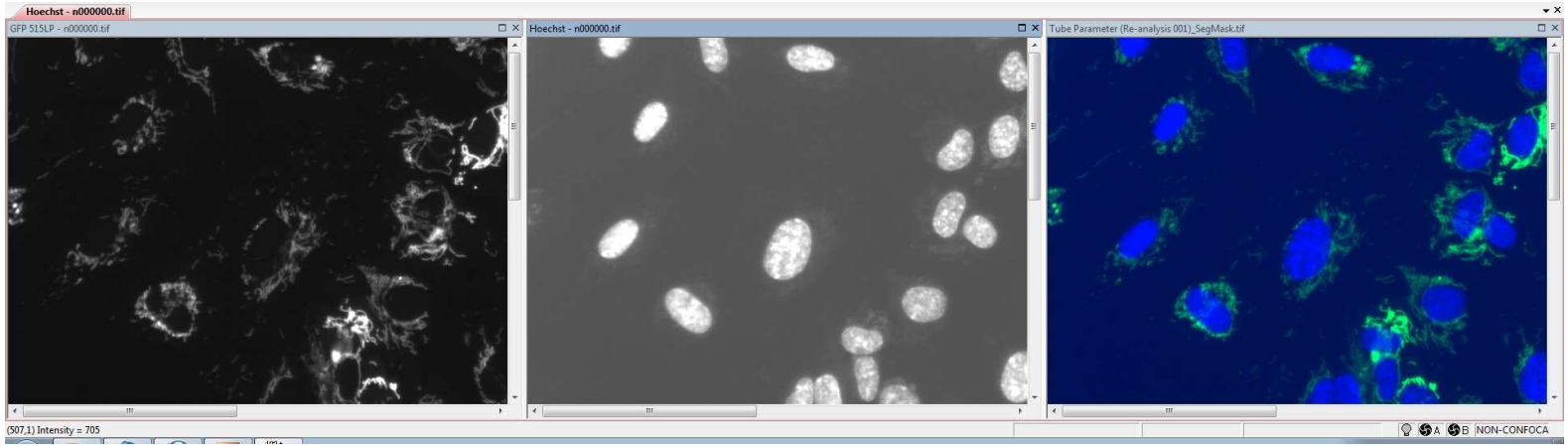


Intensity of the image can be adjusted by chosing either subtract, add, divide etc in the operation box and type in a number in the value box. Click on apply to check your settings and click OK when you are satisfied with the settings.

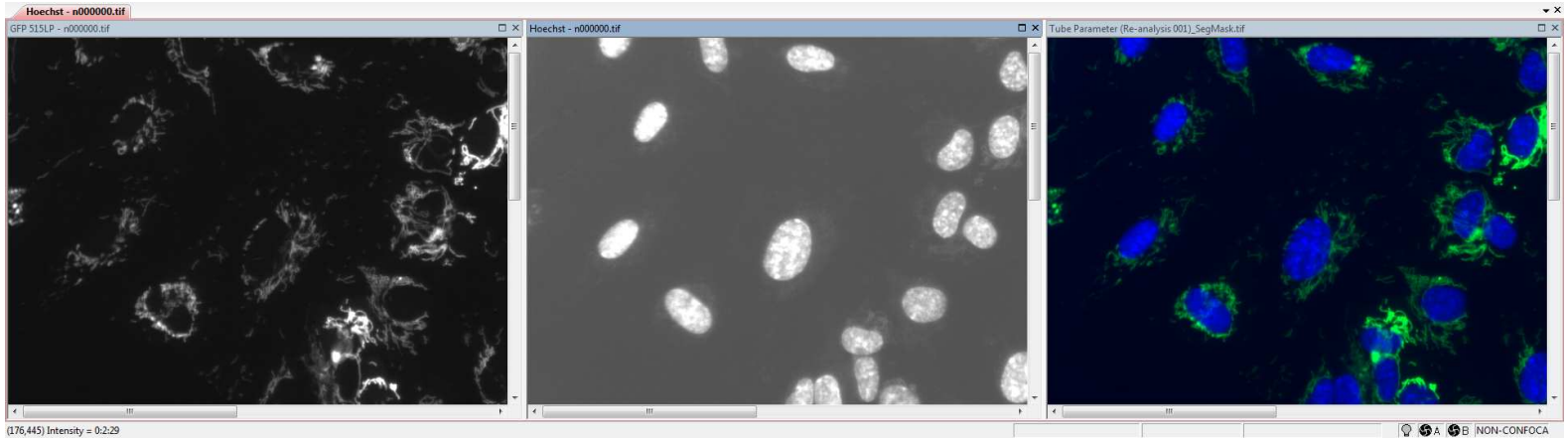
When you click ”Apply” the lower screen will appear like **Panel 1** below. If you want to clean up the image (in this example there is to high intensity in the blue channel), do as showed in the ”Image Merge” box to the right. The result is shown below in **Panel 2**.



Panel 1



Panel 2



Remember, the high-throughput imager is not meant for acquisition of pretty pictures but rather imaging of a high number of cell cultures in a short amount of time. Most importantly, the images must be of high enough quality to be quantified. Therefore, export only images representing the different conditions and not all the images on the plate since the image export operation would be far too time consuming.

Quick check list for experienced users

When you have learned the software and built a macro imaging is easy

Check list

- Is the correct objective inserted?
- Is the correct objective selected in the software?
- Adjust exposure time for the filters you want to use
- Find your macro and check that your focusing method are able to find the focal plane in the wells located the furthest away from each other
- Choose which wells that you want to image
- Import the selected wells into your macro
- Give your experiment a name and push ”Play”
- That's it!