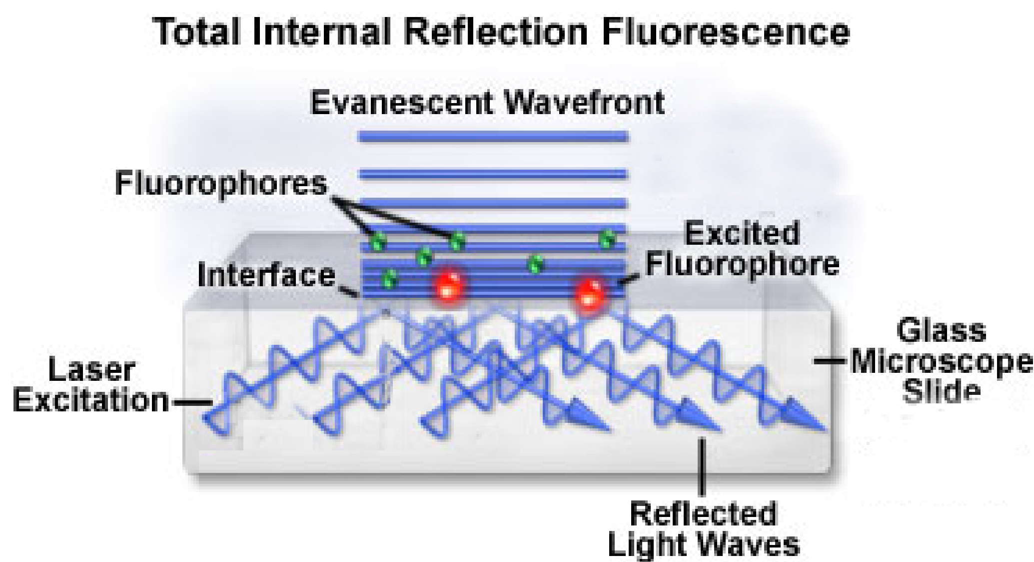
Total Internal Reflection Fluorescence microscope
Nikon Eclipse T*i*

Principal:

- Laser is pointed at an angle at specimen
- Due to this angle the laser will totally reflect
  on glass/medium interface and creates an evanescent wave above the glass
- Thickness of wave is around 200nm, but decays exponentially
- Possible to only excite fluorophores in membrane, without background signal of the cytoplasm

Benefits:

- Extreme low background signal
- Perfect for membrane studies
- CCD Camera attached will provide in fast imaging



Source: NIKON

Contact

- Martijn de Gruiter Be-343 tel. 31105
- Gert-Jan Kremers Be-346 tel. 43578

Start system

- Turn on the power by the power socket (on the floor under the table)
- Switch the mercury lamp on and then ignite it.
  - If not needed don't turn the mercury lamp on
  - The unit on the shelf closest to the door
- Turn on all devices with a button on the shelf and the table.
  - Camera and laser box are automatically enabled
- Next turn on the device with a key: Laser control panel
- Start the computer and start the program Metamorph.
  - Select suited user account.

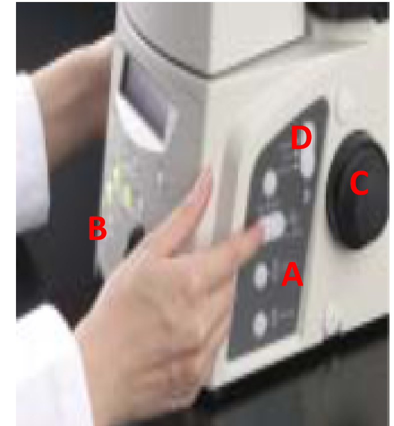
Live Cell imaging:

- When imaging at 37°C:
- The heating device to maintain your cells uses water.
  - fill reservoir with demineralized water up to level of CO2 tube
- Allow the system to warm up for 15-30 minutes
- Settings for the heating unit are paper on the wall, choose your settings
  - General settings for cells on a coverslip:
  - top heater 40.5 ° C
  - bath heater 38.5 ° C
  - stage heater 38.5 ° C
  - lens heater 37 ° C

- Ensure that the cells are grown on a glass coverslip
- Place the slide in the metal ring, tighten firmly but not too hard because it will break the glass
- Add 1-2 ml of the medium from the appropriate well. There is a convex surface
- Move with a paper towel around the edge of the coverslip from below with a little pressure, if no medium leaks from the ring your seal is good.
- Wipe clean the underside of the slide with ethanol to prevent contamination of the objective and microscope

Finding focus

- Put the objective in the lowest position using button A
  - Lowest position is ~500 um
- Choose the objective with the right magnification
- Put a little drop emersion oil on the lens
  - Only if it's an oil emersion objective
- Place the metal ring in the middle of the heating chamber
  - Secure with the metal clips
- Centre you cells above the objective with the joystick
  - Button on top of joystick will change speed, S (slow) or F (fast)
- Turn button C until the oil is in contact with the coverslip
- In MetaMorphs Taskbar choose "transmission to eyes" to illuminate the cells with the halogen lamp
- With button D you can use the fine adjustment control knob
  - course, fine or extra fine



- Look through the eyepiece and bring cells in focus.
  - The display of the microscope shows the height of the objective
- Finding appropriate fluorescence cells can be done with the mercury lamp
  - Choose the illumination settings for your fluorophore in the Taskbar in Metamorph
  - Increase or decrease brightness by changing the ND filters
  - ND 4, 8, 10 will block intensity, the can be combined, use minimal intensity to reduce bleaching of your fluorophore
- When your sample is in focus you can focus the beam of the laser if necessary, for detailed explanation ask a designated OIC member
- To change coverslips, bring objective down with lowest button A. Bring objective back to previous height with upper button A

Perfect Focus System (PFS)

- If focus is found a LED (E) will turn orange and/or you will hear a beep
  - Focus can be kept using the Perfect Focus system
  - Press the green blinking button E

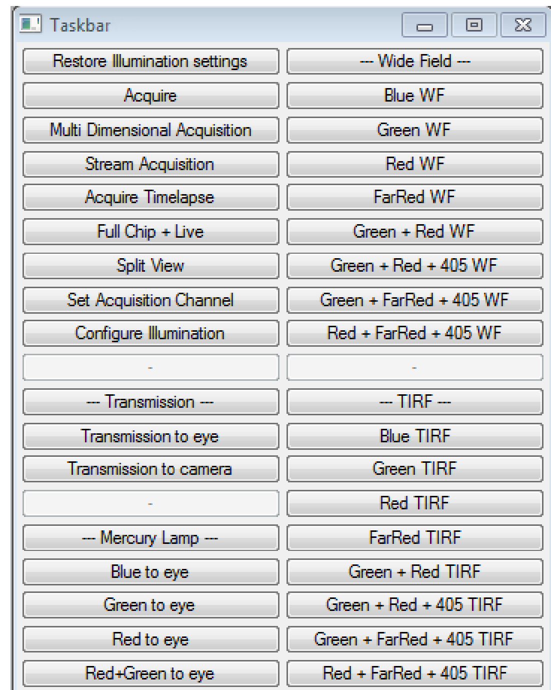
- The focus of the PFS is adjustable with a module as shown opposite
- The turntable navigate the lens up and down as shown and with the blue button toggles between fine (pressed) and coarse
- Focusing with perfect focus should be viewed through the camera

The buttons FGH give the direction of the output signal:

F: eyepiece G: camera left QuantEM:512SC H: camera port right

Metamorph - Taskbar

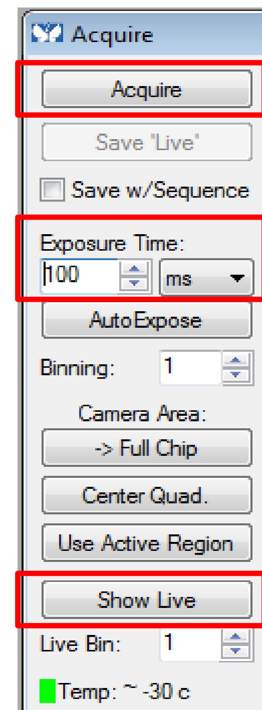
- On startup your personal taskbar is loaded
- Metamorph functions can be accessed easily from a Taskbar and an
  Acquire window
- You can restore illumination settings with the button: "Restore OIC settings"
- Open the Acquire window with Acquire
- Access Acquisition modes with: MDA & Stream
- Select preferred illumination settings
  - Left side: lasers for camera,
  - right side: mercury lamp or transmission to eyepiece
- Open FRAP or laser console



Metamorph - Acquire window

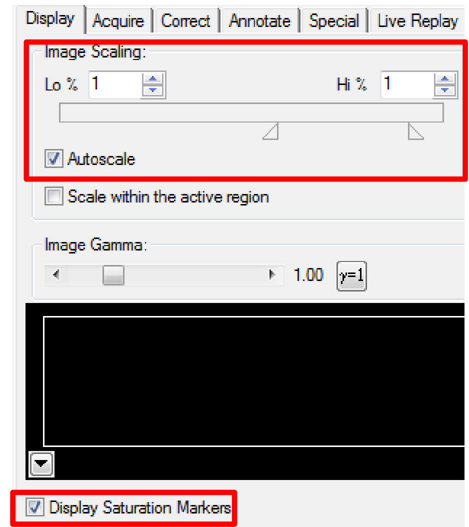
Sidebar left:

- Exposure time
  - Minimum interval time at full chip is 90ms, quad chip is 50ms
  - Exposure time can be lower than interval time
- Full Chip, Quad Chip, Use Active Region
  - Use full chip, middle quadrant, or region selected by ROI
- Live bin & bin
  - Combine pixels to increase readout speed
  - Use only for live viewing or real recording
  - Normal state = 1
- Start Live/Stop Live
  - Start/stop live mode



Acquire images

- To acquire live mode press "Acquire" button
- Separate images can be recorded as separate images or in stack

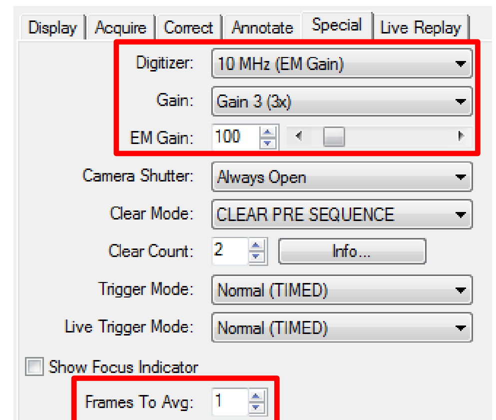


Tab: Display:

- Auto scale option
  - Enable Auto scale or change custom auto scale
  - Full bit-range is saved to file, this is only for visualization in the software
- Visualize saturated pixels

Tab Acquire:

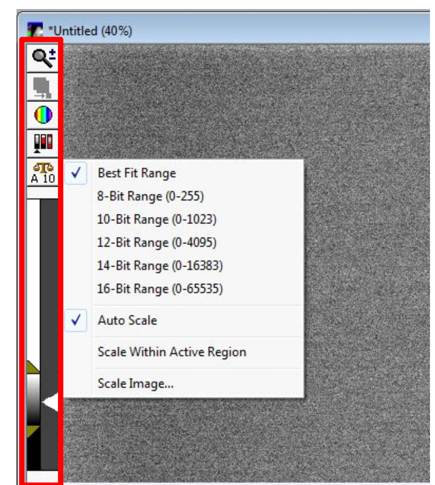
- Be sure to set the Illumination choice to "Current shutter"



Tab Special:

- Gain options:
- 10 MHz (EM gain), gain3 3x is standard setting
- Control gain between 0 and 1000
- Use "Frames To Avg" option to average images / or use longer exposure time

Live image window

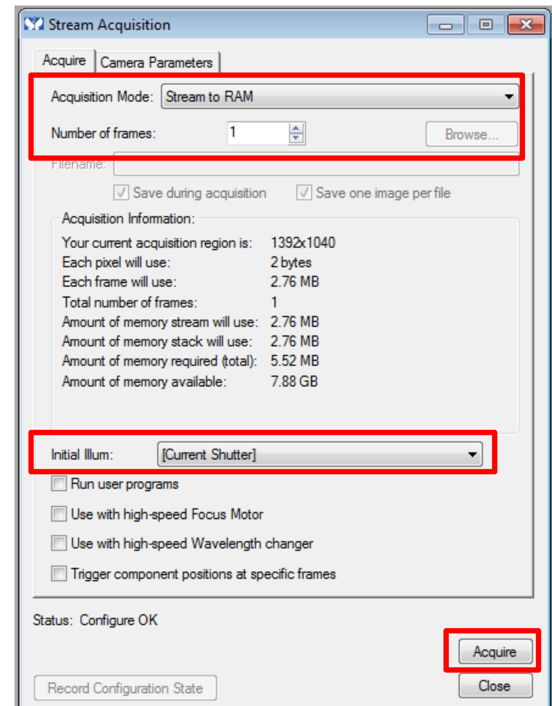


- Magnifying glass
- Display image in other LUT
  - Grayscale, Color by wavelength
- Histogram of intensities
  - Manual scaling can be done with the orange arrows
- Auto scaling options
  - Best fit range, 8/12/14/16 bit
  - Auto scale on/off
  - Scale within region

Streaming imaging

Open the Stream Acquisition window with the button in the taskbar

- Start recording with Acquire button
- Check if chosen configuration is ok



Tab Acquire:

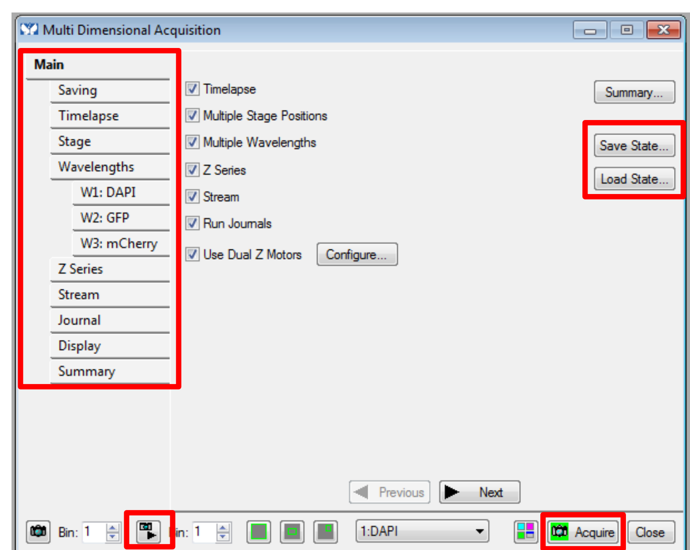
- Acquisition modes:
  - RAM save later to hard disk
  - HD choose name and location to save
Save as on file or separate TIF files
- Enter the desired number of frames
- Select "Initial illumination": *Current Shutter*

Tab Camera Parameters:

- Recording options:
  - Average frames
  - Number of frames to skip, during acquisition
  - Don't show recording (faster when exposure time < minimal interval time)
- Camera readout default options should be:
  - Camera state: HALT
  - Shutter mode: OPEN PRE SEQUENCE
  - Clear mode: CLEAR PRE SEQUENCE

Multi-Dimensional Acquisition (MDA)

Combine multiple tasks: z-stack, time-lapse, streaming, multiple positions, multiple wavelengths, use of journals.



- Open the MDA window using Taskbar
- Select acquisition mode(s) in *Main*
- Continue with the tabs shown left:
  - Saving: select filename and folder
  - Select illumination settings
  - … options for acquisition modes
- When setup is done press acquire
- Save or load settings of experiment setup, with Save/Load State buttons
- Live mode can be started from MDA window

MDA: Time lapse imaging

- Activate the time-lapse option in MDA's main window
- Set the desired interval and time
- Choose the desired illumination settings in the wavelength tab
  - Or multiple when the option multiple wavelengths are selected
- Start recording by clicking on Acquire

Shut down system

- For live cell imaging:
  - Remove Tokai Hit incubator from the stage and place it on the table
  - Close the Tokai Hit incubator with a plastic well
- **Clean objective carefully**
  - Remove oil with lens paper:
  - Hold lens paper tight between your fingers and wipe over objective
  - Clean lens with 2-propanol on your lens paper
- Cover stage with lens paper box
- Save data to OIC network storage (O:\ drive)
  - To collect the data from your own pc, connect to network storage drive via the address: \\oic-station\oic
    user: guest, password: guest
  - When the hard disk of the microscopes pc gets full, data will be deleted
  - You are responsible for your own data, the networkdrive is only for transport

- **If you are the last user of the day**
- Shut down all devices
  - Shut down mercury lamp as last
  - Camera power is switched at the adapter box at the floor
- Switch off the power socket located at the floor

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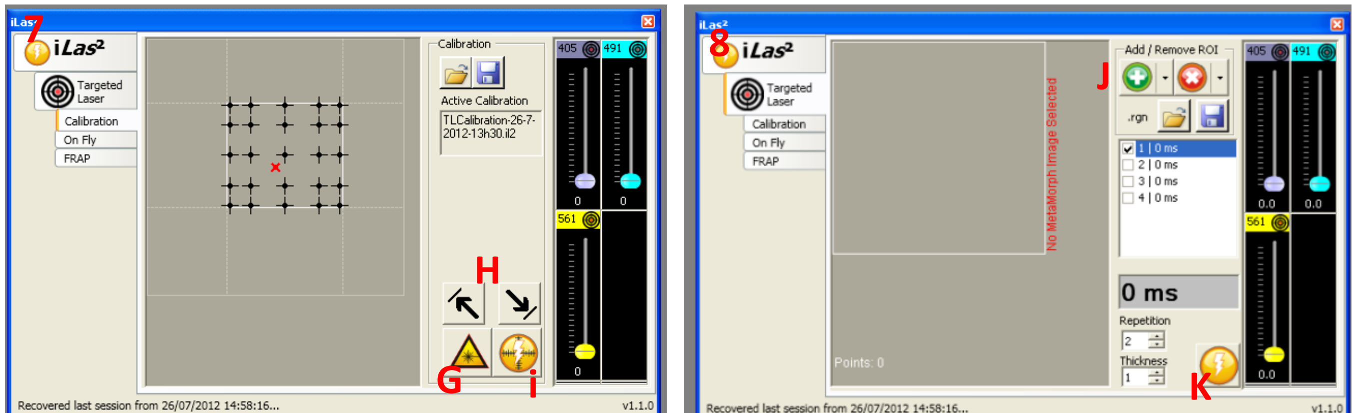
FRAP on the Fly Calibration

Before you can use FRAP the laser must be calibrated



- Find an empty piece of glass without cells/fluorescence
- Set the exposure time around 100 - 200ms
- Open ILAS2 with icon
- Open calibration tab and put the power of selected laser to 100% (in ILAS2 window)
- Activate the laser [G] and press " Show Live"
- Place laser in upper left corner by moving the red cross, press [H]
- Repeat for lower right corner
    - If the spot isn't visible or too big and bright change power and/or exposure time
    - view is rotated, so move laser in other direction
    - keep in mind to move red cross into left and right corners of the calibration screen
- Calibrate with button [i]

- To check if your calibration was successful open " on the fly" tab
- Change time to 3000
- Increase the power of your laser and press " Show Live"
- Click in the image and check if laser pulse in at the position of your mouse pointer

Troubleshooting - FRAP

<u>FRAP calibration error</u>

- Notification: Calibration canceled (exceeded 15 bits)

Possible errors

- Emitting fluorophore or auto fluorescence
    - Ensure that the area of the slide really empty
- Too much laser power or exposure time
    - Reduce laser power in the ILAS2 window and/or exposure time in Acquire window