



## Rescan Confocal Microscope, model 1 by Confocal.nl

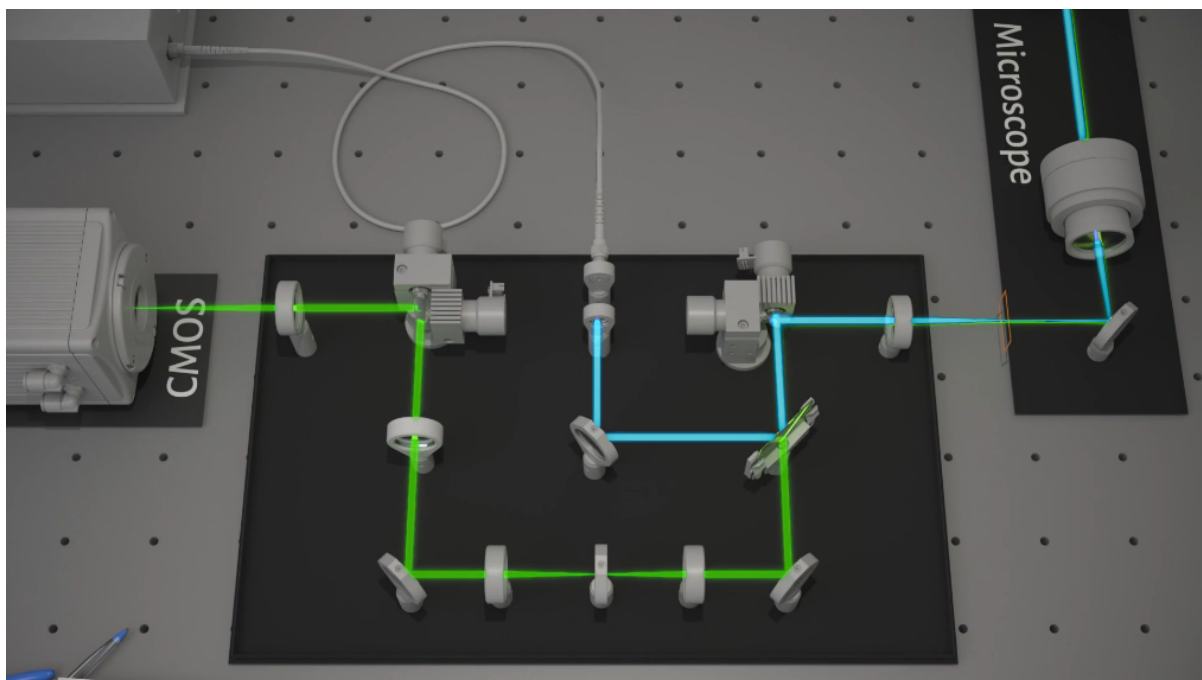
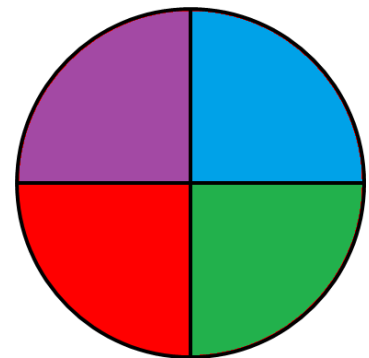
Nikon Eclipse Ti

### Principal:

- A confocal microscope with a camera instead of a detector.
- By enlarging the scan with a second set of scanning mirrors, an increased image is projected on the camera
- With this increased projection on the camera, a higher resolution is gained.
- Increased resolution of  $\sqrt{2}$  in the lateral dimension
- Church window 4 channel emission filter,  $\frac{1}{4}$  of the camera is used per channel in the emission filter.

### Benefits:

- $\sqrt{2}$  better lateral resolution
- Optical sectioning of a confocal microscope by a pinhole.
- Up to 4 channels



Optical light path of the RCM, adapted from [www.confocal.nl](http://www.confocal.nl)

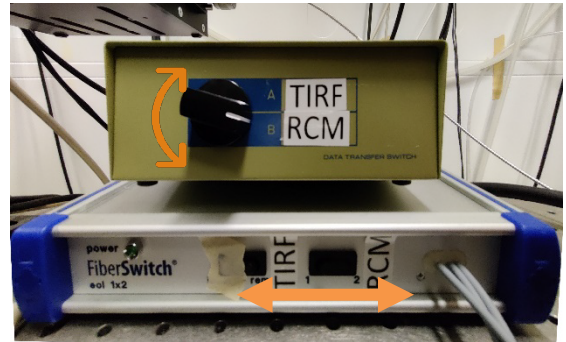
*Re-scan confocal microscopy: scanning twice for better resolution*

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## Startup

- Switch the DAC switch & Fiber switch to RCM
  - Located at the right back corner of the microscope table
  - At the end of your session: Set switches back to TIRF *after* powering off all devices.
- Switch on the power socket located on the floor
  - Laser unit is connected directly to the power socket
- Start devices needed for RCM
  - PC, located under the microscope table
  - Set USB switch to RCM
    - Button located on top of PC
  - Switch on the mercury lamp if you need to see fluorescence through the eyepieces
    - Located on the left side of the shelf on the wall
    - Switch on, wait 5-10 seconds, press ignite until orange LED is stably on
  - Transmission light, located next to mercury lamp
  - RCM controller
    - Located under the table, left side
  - Switch on the power supply unit of the camera
    - Hamamatsu Orca Flash is located at left of the orange RCM box.
  - Continue with Nikon microscope stand, Sutter Lambda control box
- NEXT turn on the devices with key (laser control panel)
  - Enable with key, activate necessary lasers
- Switch to **EPI** and de-activate **CNTL DIA** on the Nikon control unit (TI-RCP)



## 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:
  - top heater      40.5 °C              stage heater 37 °C
  - lens heater     37 °C                bath heater 38 °C

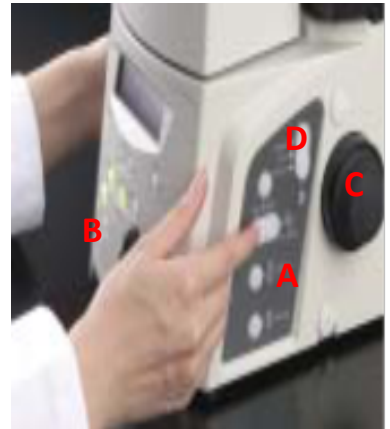
## Prepare Atto-Fluor imaging ring

- 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 1.5 ml of the medium from the appropriate well. There is a convex surface
- Let a new coverslip slowly fall over and press gently with tweezers.
- Make sure that no (large) bubbles will appear in the medium
- 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  $\mu\text{m}$
- Choose the objective with the desired magnification in the software via **objective** (figure next page, **area 6**)
  - Make sure the lens heating is not (yet) attached to an objective
- Put a little drop emersion oil on the lens (only for 60x and 100x)
- 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 changes speed, S(slow) or F(fast)
- Turn button C until the oil is in contact with the coverslip
- Via the software set Mode to **Transmission** (figure next page, **area 6**) to detect the cells with the halogen lamp
  - Adjust brightes with dimmer on left side of microscope stand
- 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 stand shows the height of the objective
- Finding appropriate fluorescence cells can be done with the mercury lamp
  - Via the software set Mode to the desired **color** (figure next page, **area 6**) to set the desired filter set.
  - Increase or decrease brightness by changing the ND filters
  - ND 4, 8, 10 will block intensity, they can be combined
  - Use minimal intensity and exposure to reduce bleaching of your fluorophore
- When changing coverslips, bring objective down with lowest button A. Bring objective back to previous height with upper button A



The buttons FGH give the direction of the output signal:

- F: eyepiece      G: camera left: RCM      H: camera right: TIRF

## Perfect Focus System (PFS)

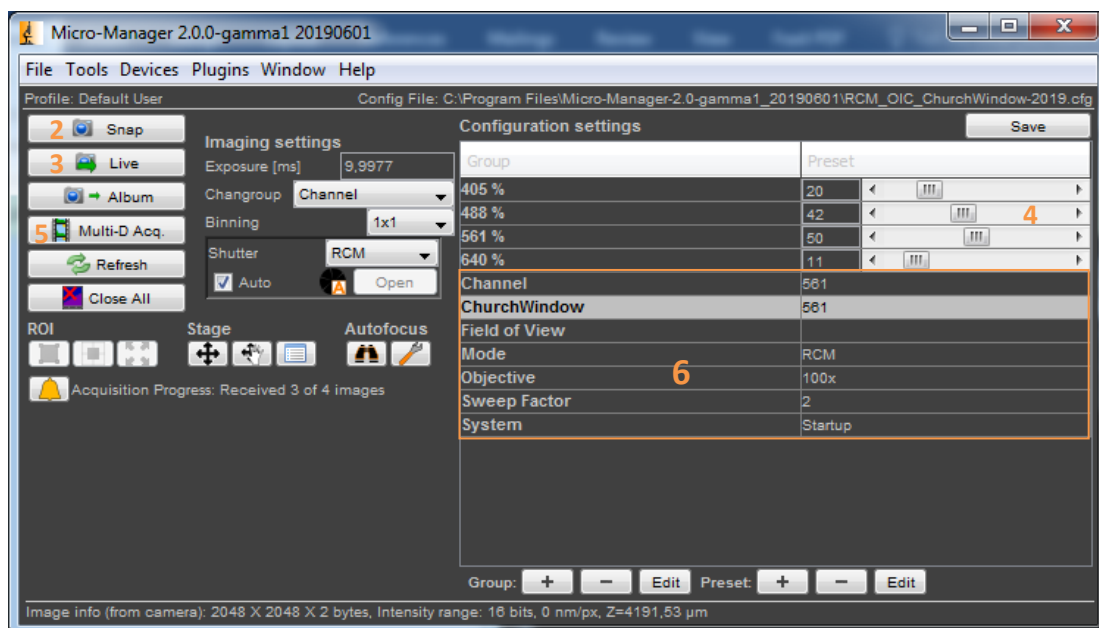
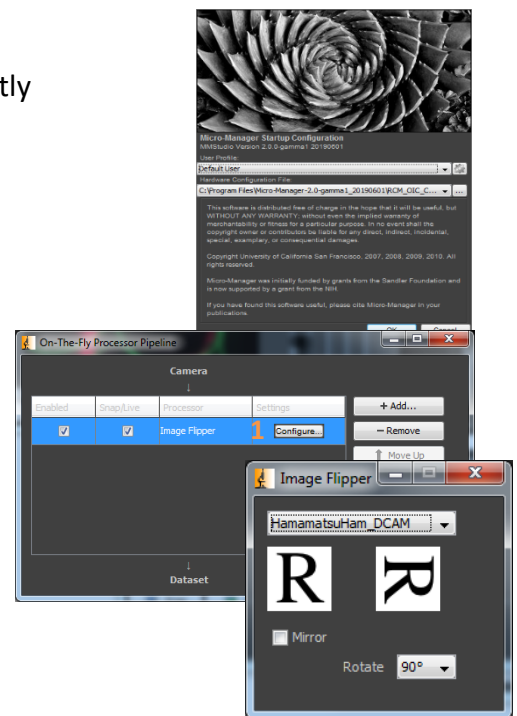
- Focus can be kept using the Perfect Focus system, if focus is found a LED (E) will turn orange and/or you will hear a beep, press the green blinking button E



- 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.

## RCM software: Micro Manager

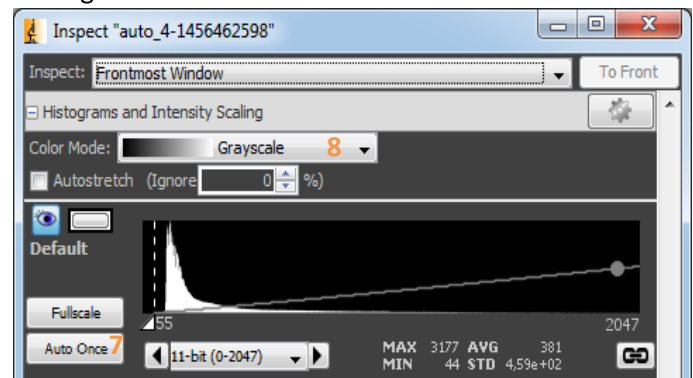
- Make sure Nikon display has EPI and DIA control set correctly (see Startup at page 2)
- Start MM-Gamma located on desktop
- Load settings (do not change file)
  - "C:\Program Files\MM\_RCM\RCM\_OIC\_ChurchWindow.cfg"
- Before you continue, set camera's orientation
  - If On-the-Fly Processor pipeline window is closed
    - Open via Plugins > On-the-fly image processing > Configure processors
  - Click on configure button (1)
  - Set camera to HamamatsuHam\_DCAM
  - Set rotate to 90°
- For a single channel acquisition:
  - Set the ChurchWindow (area 6) to the desired excitation channel (NOT CHANNEL)
  - Make sure Mode is set to RCM
  - Set sweep factor to 1 for normal confocal or 2 for Re-scan Confocal



- When a scanned vertical line is seen instead of a complete image
  - Set Field of View to 512 (6)
  - Set channel to desired excitation wavelength (6)
  - Set Church Window to same excitation wavelength as channel (6)
  - Press live (2)



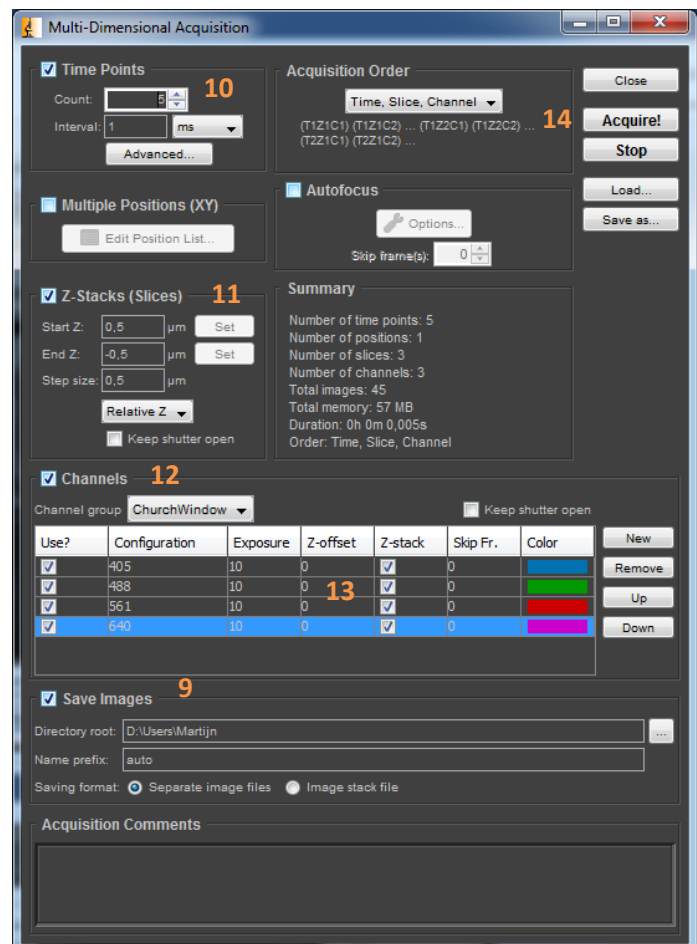
- Adjust laser power (4) to obtain optimal intensity and prevent photo bleaching
  - Open histogram via gear icon in left bottom of live image window
    - Choose Image Inspector
  - 8 bit scaling is sufficient
  - Adjust scaling with auto once (7)
  - Drag the vertical dotted line to background level
    - ~100 grayscale values
  - Adjust laser power to obtain tens of intensity values above background level.
  - Adjust Look Up table via dropdown window (8)



## Multi-Channel imaging

Combine dimensions in acquisition

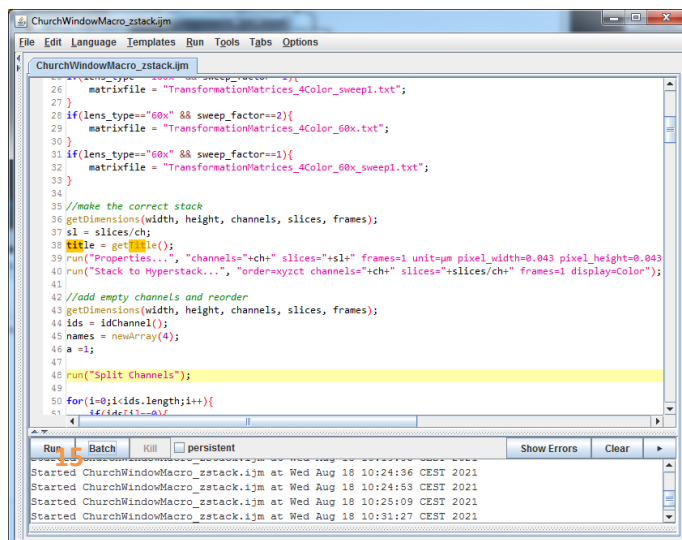
- Save data in separate image files (9)
  - Select directory and image filename
- Time (10)
  - Set interval time and frame count
  - Minimal interval is depending on future steps, check acquisition time to determine minimal timelapse interval
- Z-stacks (12)
  - When relative Z is used
  - Select travel up and down from focus plane
- Channel group
  - ChurchWindow for multi-color imaging
  - Sweep for images in confocal and RCM
  - Only one group can be chosen, not possible to combine channel groups
  - Modify separate channels in (13)
- Start acquisition, with **Acquire!** (14)
  - New filenames are created, with a new position in focus, continue with acquire and a new filename will be created.



## Image post-processing

Single images are created for every channel. With our “Church Window” emission filter, every channel uses one quadrant of the camera. To align these channels in one multichannel tiff, please use the macro:

- Open Fiji on the desktop
- Open a new Script window via File>New>Script
  - Open the macro via File>Open Recent> *ChurchWindowMacro\_auto\_v[date].ijm*
  - Or via folder *C:\ChurchwindowsSetupFiles\Macros*
- Drag folder Default from the acquired image stack to the grey bar of the Fiji main window
  - Confirm window “Open Folder”
  - Checkbox for Sort numerically must be active
- Click run (15)
- Do not interrupt Fiji while the macro runs
  - Do not click on images or change view etc.
- The final image still needs to be saved (in the root folder of the original image.)



## Contact

- |                      |        |            |
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