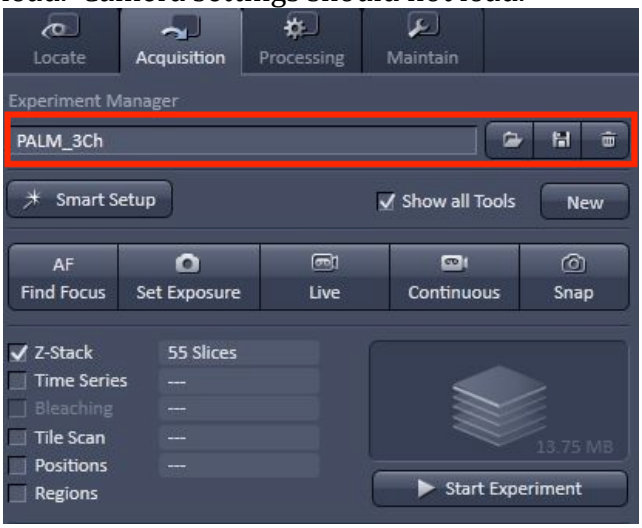
PALM/dSTORM image acquisition Protocol on the Zeiss ELYRA

System and ZEN Startup

1) Turn on "main power" and each of the "components" and "system/PC" switches.
2) When system has powered on, turn on the acquisition computer (HP Z820)
3) Login as LSM User ~~(pw: zeiss)~~
4) Start ZEN by double clicking the black circular icon (note, there can also be a blue icon)
5) Click "start system".
    a. *Monitor "boot status" for any realtime controller timeout. If there is a realtime controller timeout, push the round reset button on the front panel of the realtime PC (black computer next to the HP Z820, Lian Li computer. Note: there is a black door that covers the front panel).*

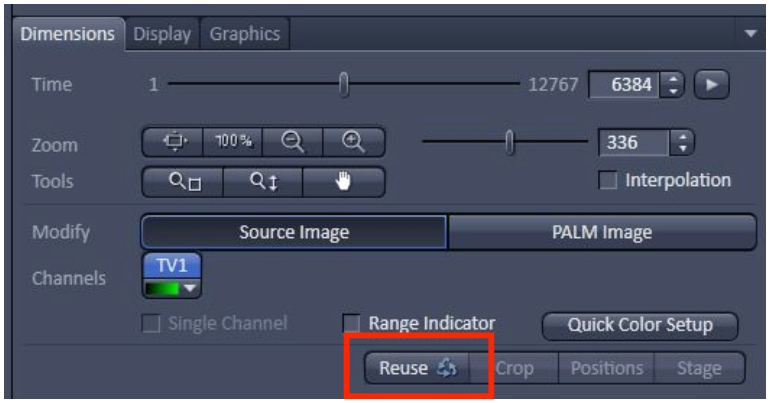
Load acquisition configuration (option 1)

1) After ZEN has started up, click the "Acquisition" tab in the upper left hand region of the display screen.
2) Find the "Experiment Manager" region immediately below the "Acquisition" tab and click the "open" icon to the right of it.
3) Find the configuration "PALM_3Ch" and select it. All relevant lasers and track configurations should load. Camera settings should not load.



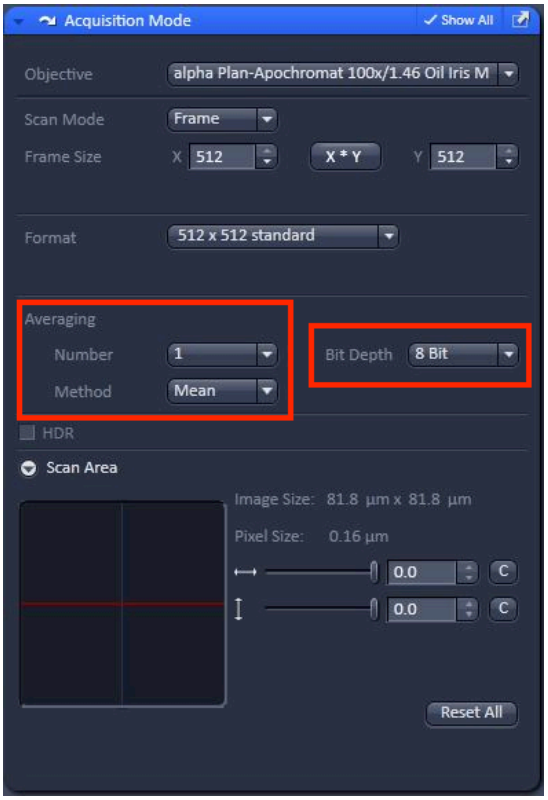
Load acquisition configuration (option 2)

1) After ZEN is started, load an image that was acquiring using settings you desire.
2) Find the "Reuse" button in the "Dimensions" tab below the image display window. Click it. This will load all the tracks, lasers, camera settings, and channel settings from that acquisition.



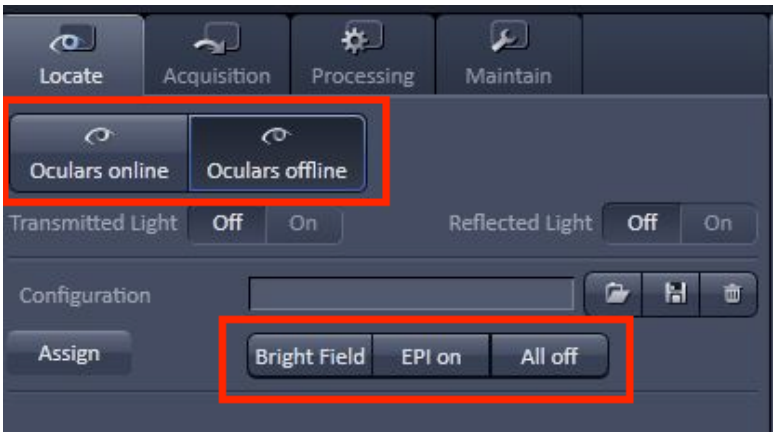
Check configurations

1) Using the touch-screen display, make sure you are on the 100x, 1.46 NA objective
2) In the blue "acquisition mode" options window (within acquisition tab), check that the following is set:
    a. 16 bit-depth images
    b. no averaging



Loading and finding Samples

1) Set to the 100x 1.46NA objective (microscope -> objectives -> position 4 on the TFT display)
2) Carefully insert the proper sample holder into the z-piezo stage.
3) Apply oil to the objective, and then insert the sample into the holder. Applying clips on top of round glass-bottom dishes is optional.
4) Raise the objective to the sample, then close all doors and sliders in the laser-safety box.
5) In ZEN, click the "locate" tab, turn "oculars online", and use either EPI or transmitted light to find your sample through the eyepieces.
6) Turn "oculars offline" when finished.

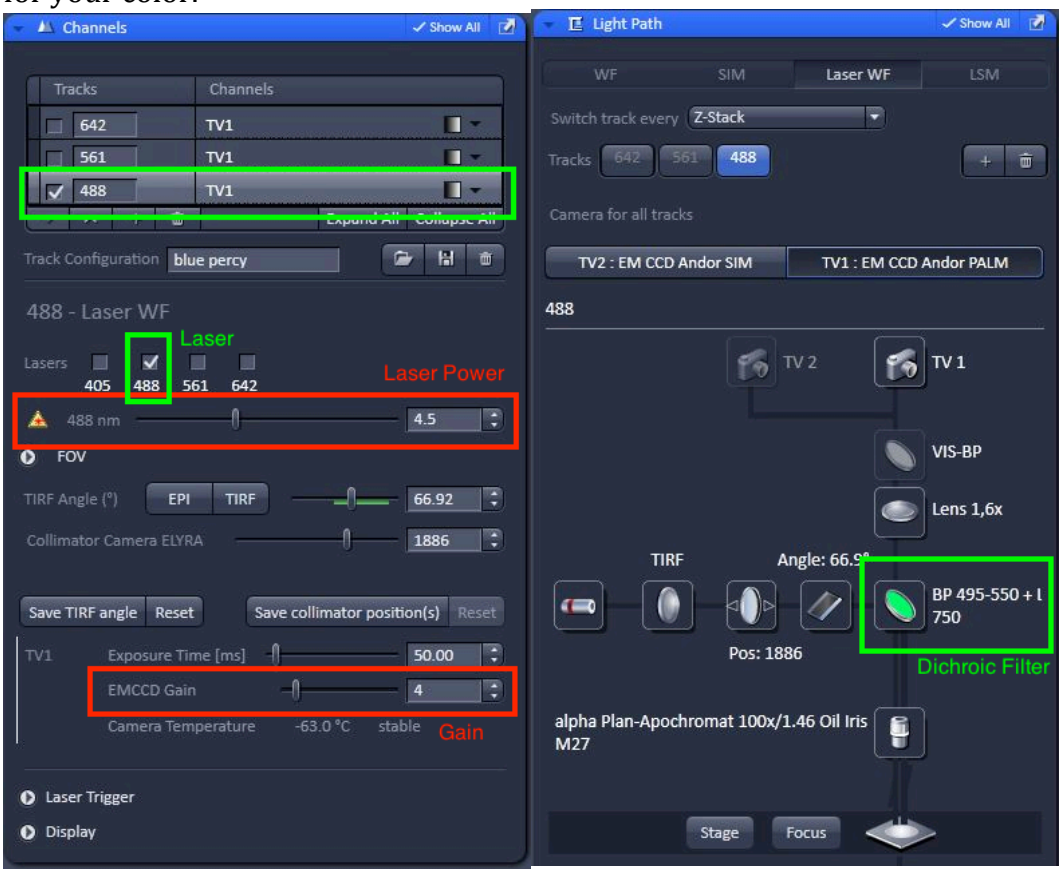


Acquiring PALM (pa-FPs) data (additional dSTORM (organic dyes) notes in blue)

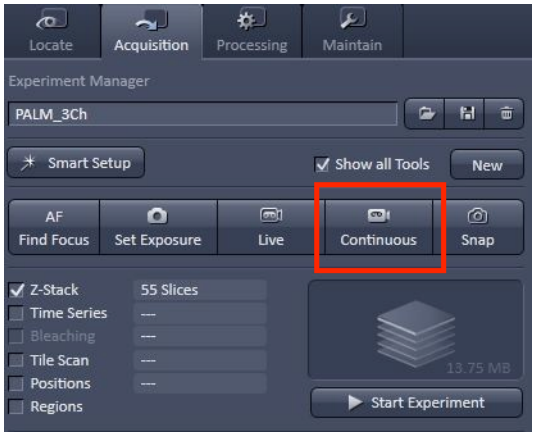
1) If you are imaging photoconvertible dyes like EOS, you can first find a cell with good expression by searching in green. If you are imaging an organic dye as in dSTORM, you can find cells with the appropriate excitation wavelength of your dye

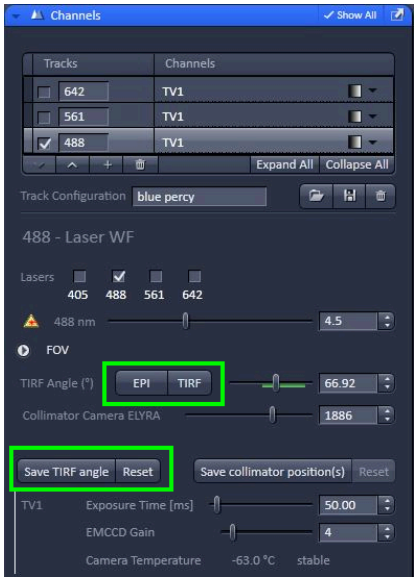
2) Apply the dSTORM buffer just before imaging. The buffer will last for 30 - 45 minutes depending on oxygen diffusion (and generation of reactive oxygen species that will bleach the dyes). You will know that the buffer has gone bad when molecules do not switch quickly or at all, or when the fraction of "on" molecules disappears quickly.

3) **Initial settings**: Under the "channels" window, start by imaging at low power (1.0-5.0%) in EPI mode. Check that there is nonzero gain and that the correct dichroic filter is selected for your color.



4) **Optimize illumination**: Using "continuous" and not "live" mode, find your sample and adjust the focus to the region of interest. Then, if your region appears to have high background, you can switch from EPI to TIRF mode. If your structure disappears, then it lies beyond the TIRF region and you will have to adjust the slider to a lower TIRF angle. Click "save TIRF angle" to have the software remember your last setting. Toggle between EPI and TIRF to reset to your saved angle.





5) **Switch to the PALM color.** (If using EOS, that means step 3 above was in 488 and now we want 561). Change the settings for this channel to those used in step 3 above (TIRF angle, camera field of view, laser field of illumination). not necessary for dSTORM

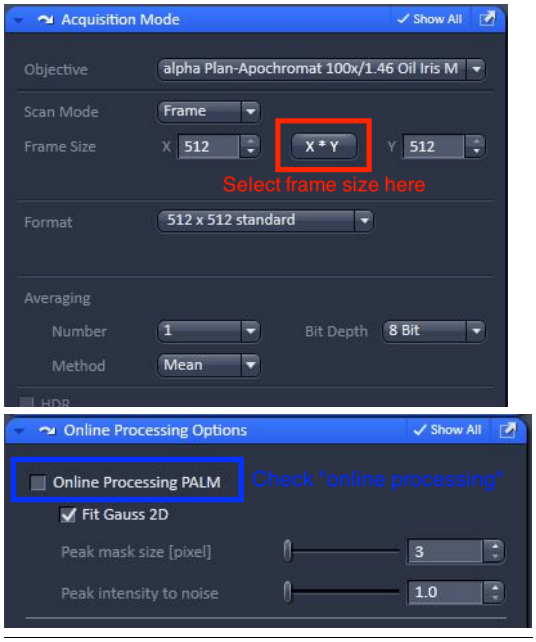
6) **Use "continuous" to check structure**. Ideally, no molecules would be converted and the image will be dark or blank. It is OK if molecules have converted. not necessary for dSTORM

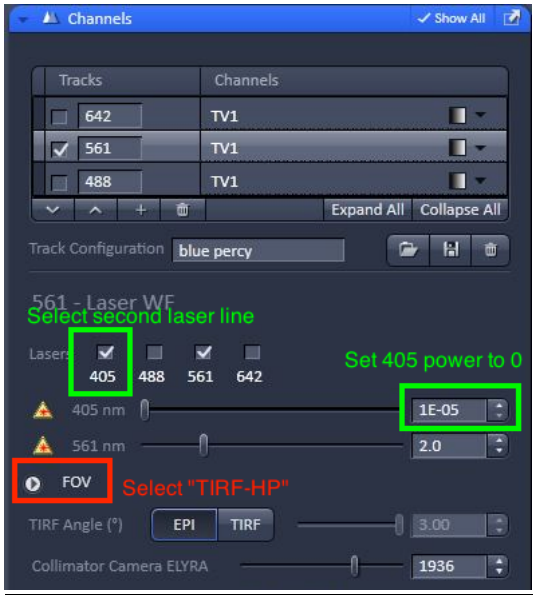
7) **Bleach the sample.** Do this by turning the illumination laser power to 100% while running "continuous". Set camera gain low (~10) to avoid saturating the camera. The purpose of this step is to "switch off" molecules that have already converted to red and will contribute to background during the real imaging. Stop "continuous" no more than 10 seconds later (you should not need more time than this to bleach). In dSTORM, bleach step may take slightly longer (~10 seconds), but ideally should complete almost immediately (less than 3 seconds).

8) **Adjust acquisition parameters**:

   PALM imaging

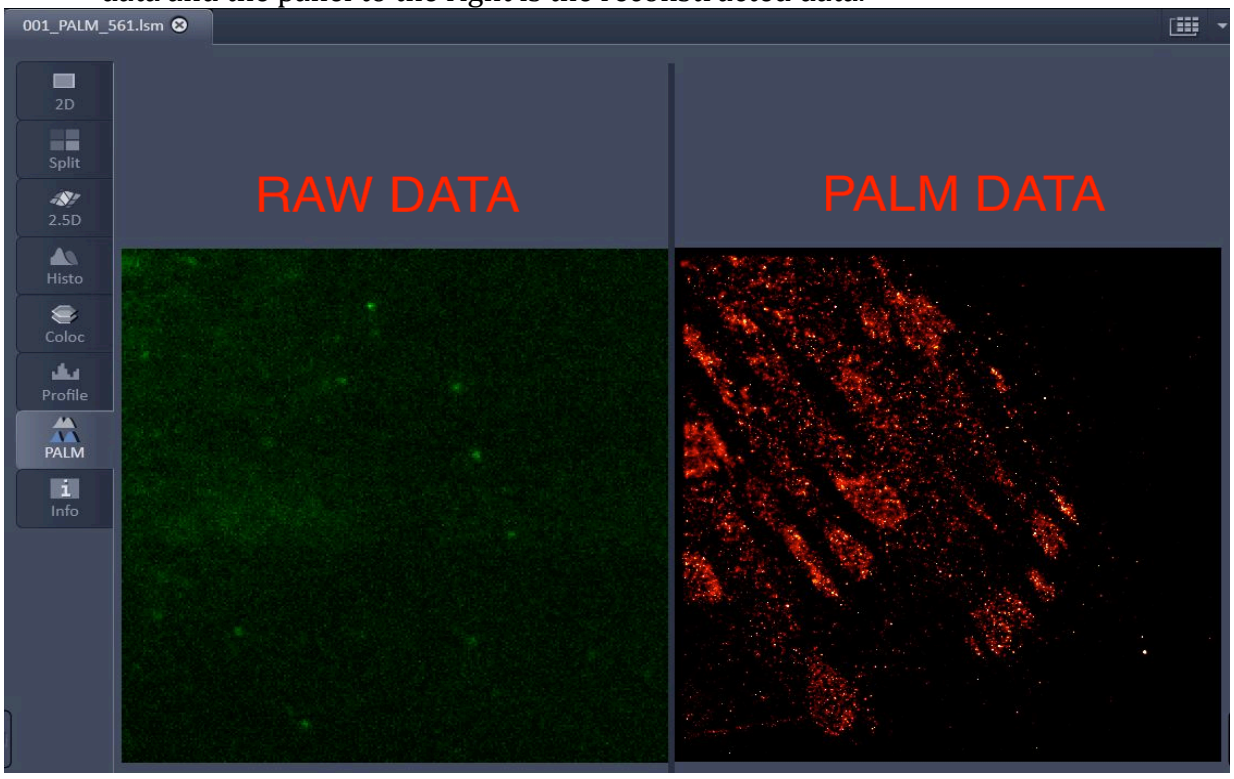
   a. Gain = 250 (reduce if image saturates)
   b. Exposure time = 33 ms (17 ms if dSTORM and TIRF_HP and 256x256 field of view)
   c. 405 laser power = 0 (software will show zero = 1e-5) (Both the imaging and 405 lasers will be checked ON. Imaging laser power will be 100 but 405 will start at zero)
   d. Click on "online processing PALM" in the "online processing options" window.
   e. Time series = 40000 frames, no interval. We can stop this early if necessary





9) Acquire! "start experiment"

a. While imaging, you should see two image panels. The panel to the left is the raw data and the panel to the right is the reconstructed data.



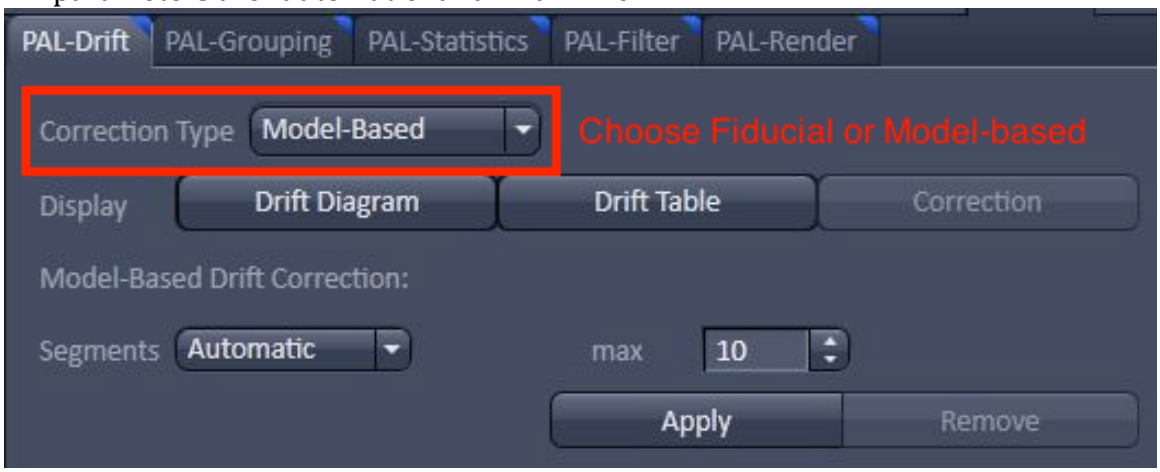
b. If PALM imaging (EOS or PA-proteins), you will have to raise the 405 laser power during imaging to convert single molecules to the red channel. Do this gradually and gently.

c. The contrast in the reconstructed data is often very high, but the relevant signal is usually contained in the lower 99% of the signal. To cut out data in the top x% (which thus appears saturated in the image), select the "PAL-render" tab below the image and set "render auto-dynamic range HR" as necessary.

10) **Save data:** make sure you have the "source" image selected. Then click file-> save data.

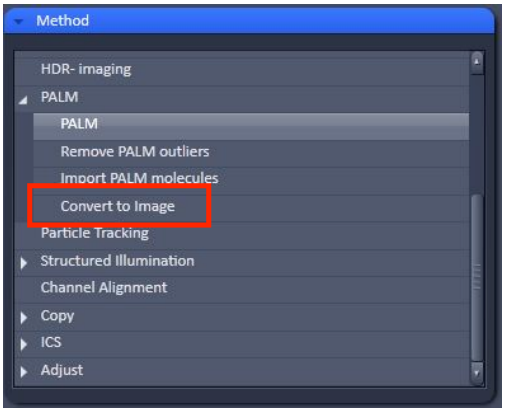
Image refinement and conversion

1) Drift correction: click the PAL-drift tab below the image (be sure you are viewing the PALM image)
   a. Fiducial-based correction: select from drop down menu and hit "Apply"
   b. Model-based correction: select from drop down menu and hit "Apply". Default parameters are "automatic" and "max = 10"



2) Grouping: click the PAL-Grouping tab below the image
   a. Max on time = 5
   b. Off gap = 0
   c. Capture radius = 0.3 pixels
   d. Hit "Apply"
3) Generating images:
   a. In the upper left corner where "Locate" and "Acquisition" options are, select "Processing"

   b. Expand the "PALM" functions
      i. Select "convert to image"
      ii. Make sure you are viewing and have selected your PALM data, then click "select" within the "convert to image" function.
      iii. Hit "Apply"



c. Save the new image in whatever format you like.

Troubleshooting

1) no image on screen
    a. check laser safety box
    b. check that you are not on eyepieces (oculars offline)
    c. check gain nonzero and laser power nonzero
    d. check that you have selected the correct dichroic
2) molecules are not switching or switching slowly
    a. for dSTORM, is the buffer fresh?
    b. is the sample opaque and/or are you imaging deep? Will need more laser power.
        i. Switch to HP = high power mode: Select "TIRF_HP" or "TIRF_uHP" in the FOV tab underneath the laser power slider. (This reduces field of view, but increases laser power density.)
    c. Are you using enough laser power?
3) Not enough molecules are appearing
    a. For PALM, are you using enough 405 power? You should require at maximum 5% 405 power.
    b. For dSTORM, have you already bleached everything? 405 can increase fraction of "ON" molecules, but it is much less sensitive. You will need to increase to at maximum 10% 405.
4) Features are not appearing or not selectable (such as FOV, save TIRF angle)
    a. Make sure you have "show all" selected for that window