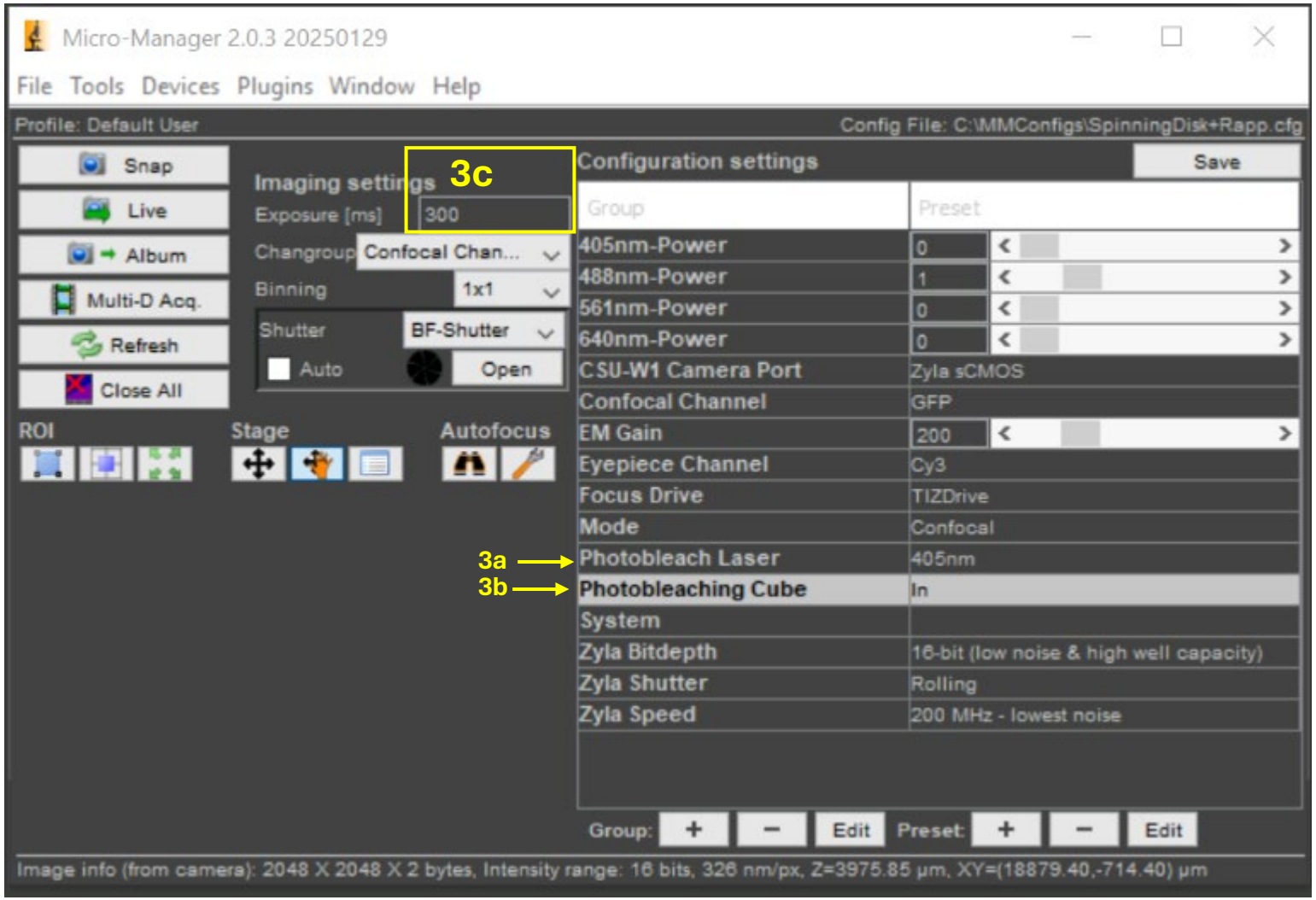
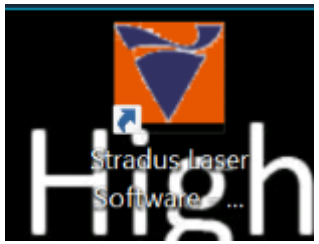
FRAP startup (Highlighted indicates a **critical** step)

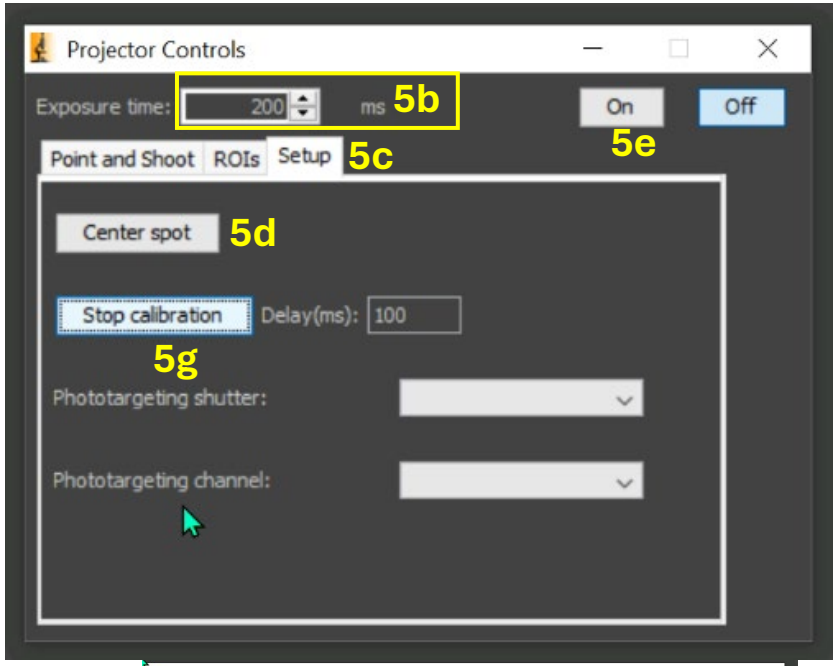
1. Power on BOTH power switches
   a) Main power (left side)
   b) Photo manipulation power (right side)
2. Open up software
   a) Micro-Manager (MM)-> Choose config "SpinningDisk+RAPP.cfg"
   b) Stradus Laser software (to control FRAP laser power)
3. Setting in the MM main window
   a) Photo Bleach laser -> Choose **405nm** or **473nm**
   b) Photobleaching cube -> **IN**
   c) Channel -> GFP
      i. 488 laser power -> 0.25 V
      ii. Exposure **300ms**
4. Prepare a FITC slide and put it on the microscope.
   a) TIP: Use perfect focus to find focus (can be done on Brightfield or by GFP/FITC)
   b) Fine to focus by focusing on the edge of a bubble
      i. Bubbles don't fluoresce! So you can make the edge sharp
      ii. Move away from the bubble to have a flat dye field

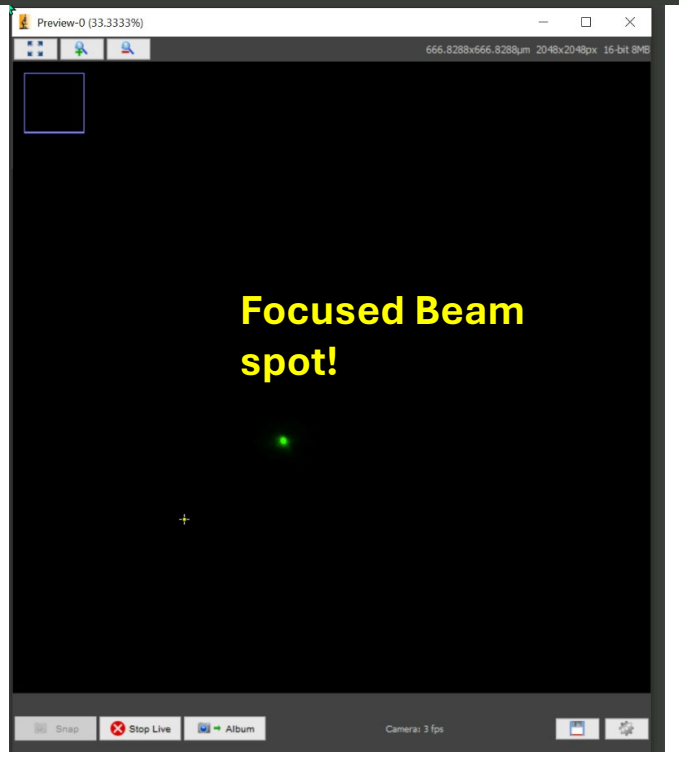


2b.
On desktop
(see slide further down for tips on control!)



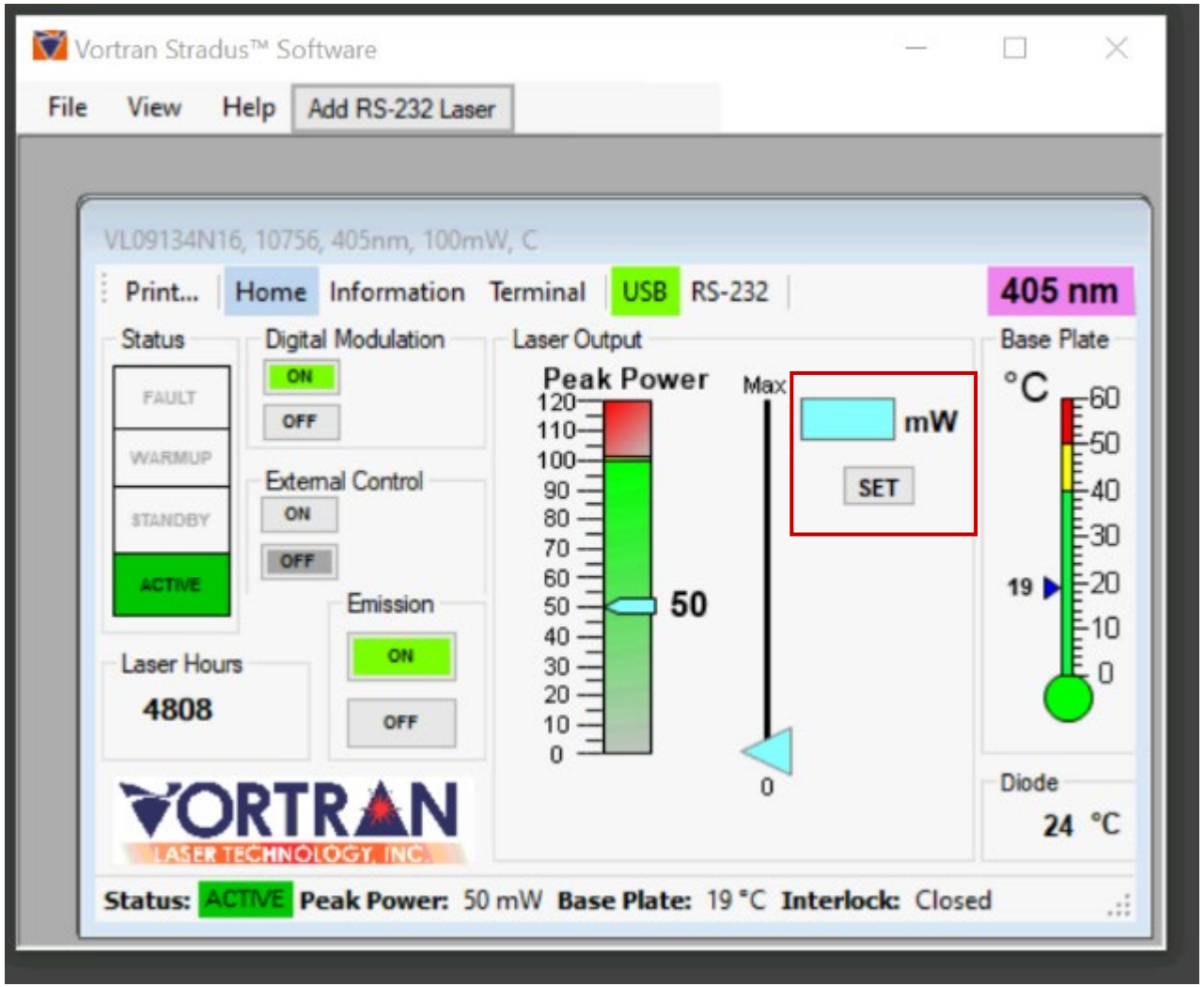
5. Photobleaching control window (called Projector)
    a) Open Projector window: Plugins -> Devices -> Projector
    b) Set exposure to **200ms**
    c) Click on the “Set up” tab
    d) Click “Center spot”
        a) You should see laser flash in a spot (will not be centered in the screen)
    e) Click “On”
    f) Use the appropriate focus knob to make the beam as tight as possible, *no more than a quarter turn is usually needed*.
        a) 405nm knob is directly behind the microscope
        b) 473nm is to the left
    g) Click “Calibrate”
        a) Beam will walk around the screen
        b) If there are no errors, then when it stops, it should be calibrated! Test with point and shoot

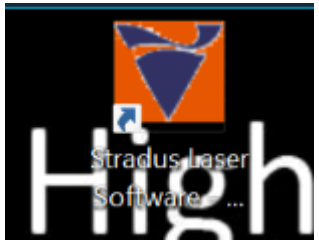




Controlling FRAP laser power

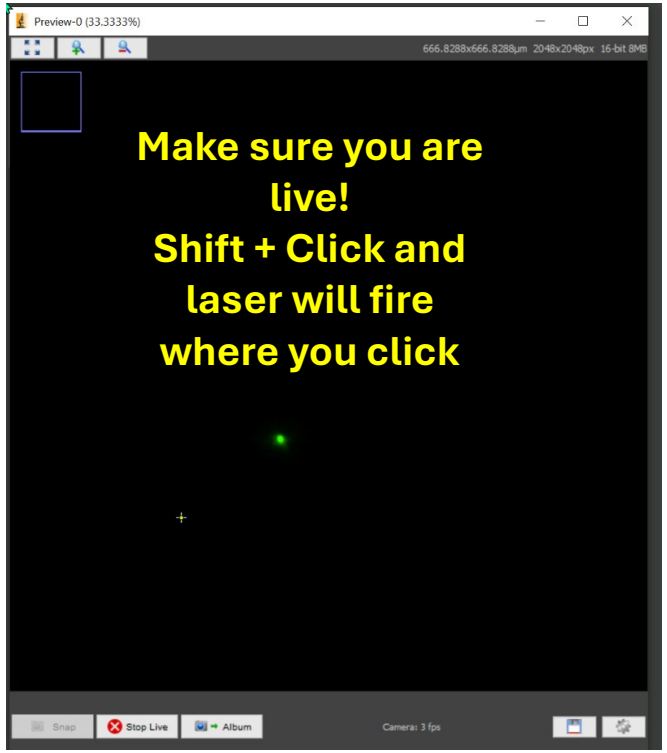
1. Recommendations- Click on the laser you want to bring it to the front, shrink the box down so it is the only one you see, this will make it so you don't accidentally change the wrong laser.
2. To change power:
    1. Type desired mW into cyan box
    2. Click "SET"
    3. NOTE: power will NOT change by dragging



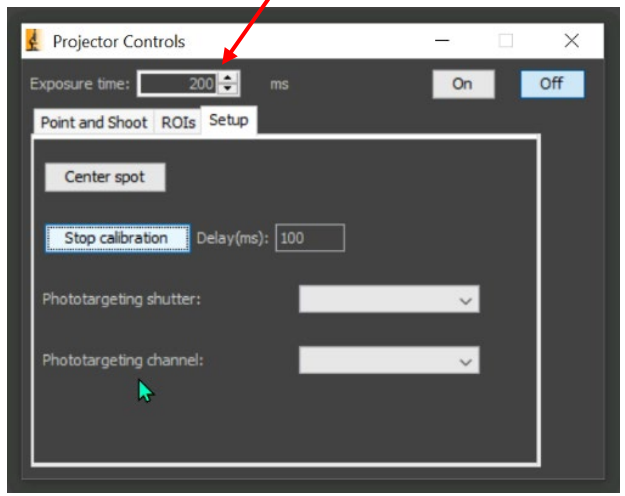


Point and shoot

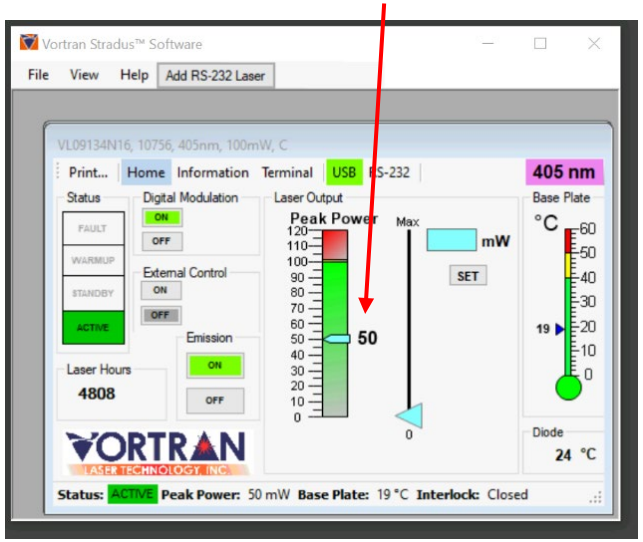
1. In the projector window
   a) Select “Point and shoot” tab
   b) Click “On”
   c) Hold down shift + click on locations in your image to bleach the desired location
   d) Important Notes:
      i. **Strength/Power** of the laser spot is determined by setting the power in the Stradus laser software
      ii. **Length of time** is determined by exposure in the Projector controls
   e) Can be run during imaging!
      a) Be prepared with the point and shoot ready to go (On and ready to shift+ click!)
      b) Set up a time-lapse in the MDA window
      c) Click “Acquire” then shift + Click to bleach while acquiring



Length of RAPP spot exposure

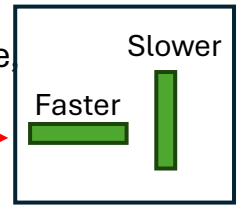


Power of RAPP spot exposure

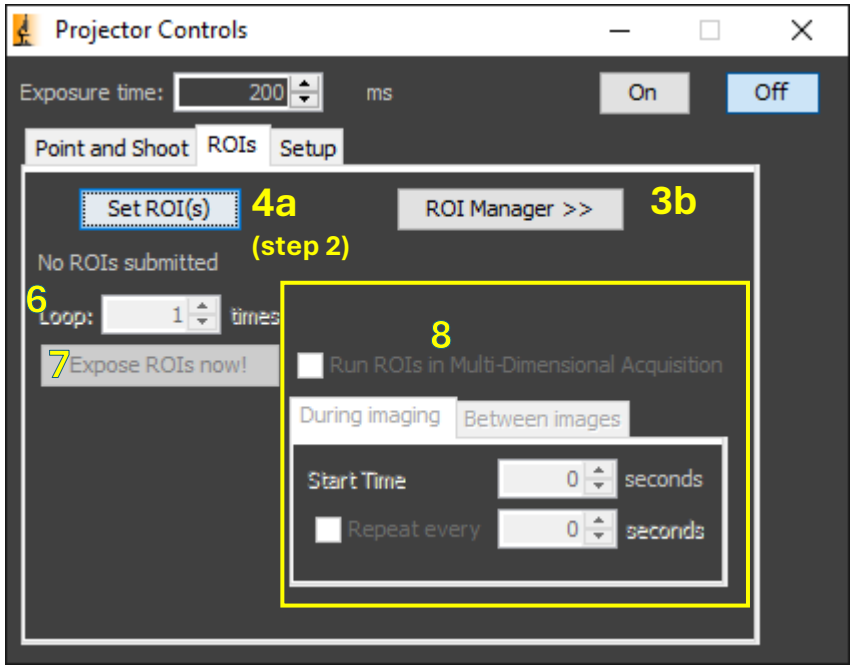


ROIs

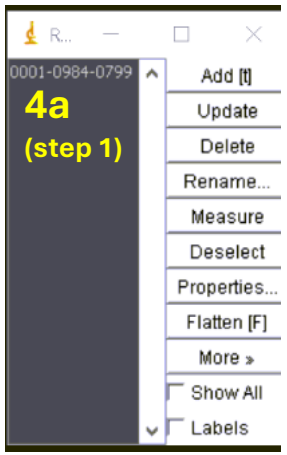
1. In the projector window
   a) Select “ROIs” tab
2. Make shape
   a) Select the shape in Image J Tool Bar
   b) Draw shape
      i. TIP: the mirrors move faster in the X direction; therefore, Longer horizontal boxes take less time to bleach than vertical ones!



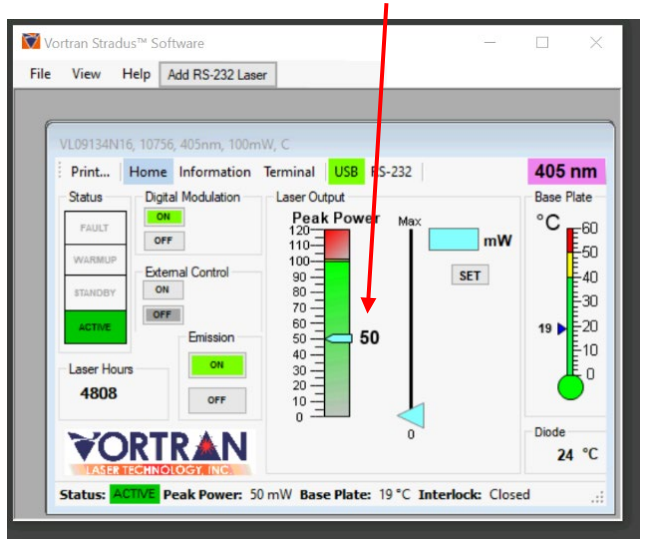
3. Transfer to ROI Manager
   a) Can type ‘t’
   b) Alternatively go to ROIs tab -> open ROI manager -> “Add (t)”
   c) TIP: Can go to More-> “Save…” to save spots for analysis later
4. Transfer ROI to Projector
   a) Highlight ROIs in ROI manager -> “Set ROIs”
   b) NOTE: *if you make new ROIs but do not set them, you will bleach your previous ROI regions*
5. **Power** for laser -> set in Stradus Laser software!
6. **Loop** -> the number of times the laser will pass over the same region!
   1. A great way to bleach more and be gentle without increasing power
   2. NOTE: *The Exposure time in the projector DOES NOT affect ROIs*
7. **Expose ROIS now** -> runs when you click the button!
8. Check box “**Run ROIs in Multi-Dimensional Acquisition**”
   1. Allows you to Set ROIs and then run them after a certain time (During imaging tab) or between Frames (Between images). NOTE: *imaging will pause while it bleaches.*
   2. A common tactic is to collect 3-5 base images in the timelapse, then FRAP and collect more



ROI Manager



Power of RAPP ROI exposure



Tips for starting FRAP

1. You will need to hone in on your laser power and loops (ROIs) or RAPP laser exposure time (point and shoot) for your specific sample
2. It is best to start with looking at this with quick bleaching while the camera is on live and testing out different parameters
    a) No bleaching? Increase laser power/exposure OR loops (be a good scientist and do one at a time!)
    b) Remember loops tend to be more gentle, but take longer, so it will depend on your sample and how fast your dynamics are!
3. When capturing the Time points early on, start with smaller intervals
    a) An interval of '0' will go as fast as possible!
    b) Small intervals -> less likely to miss fast events!
    c) Make the intervals larger if you are capturing to much data or bleaching too much without seeing much change
    d) Also start with many more time points than you will need.
        a) You can always click stop early if you are seeing no change or saw your change already.
4. Unchecking the channels will image the channel you are on as fast as possible!
    1. If you need multiple channels you can check this, but remember it will slow down your imaging
    2. Multiple Channels is recommended for after settings have been narrowed down but not at the beginning -> some exceptions like such as when doing photo conversion!
5. Don't forget to Save!
    a) Clicking Save images automatically saves your data!