Two Photon

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Theory

Pointing you to the two photon microscopy wikipedia page is about the best I can do.

Intensity Measurement

Internal Solutions

Preparing A594 Freezer Stock

- Package contains 1mg (MW 819.85)
- Resuspend in 246uL
- Place 20uL into each of 12 tubes (each has 0.081300813 mg)
- Desiccate, store at -20ºC as 32x stock
- Resuspend one tube into 330uL
- Place 10uL into each of 32 tubes (each has 2.463661 ug)
- Desiccate, store as internal stock

Resuspend in 100uL internal solution (target ~30uMol,this becomes 30.05uMol)

Preparing Fluo-5 Freezer Stock

- Package contains 500ug (MW 931.0511)
- Resuspend in 200uL
- Place 10uL into each of 18 tubes (each has 25 ug)
- Desiccate, store at -20ºC

Resuspend in 100uL internal solution (target ~300uMol, this becomes 298.3uMol)

Preparing Calibration Solutions

- create 1M $CaCl_2$ (1.470g $CaCl_2$ / 10 mL H_2O)

make concentrations (in mM) of: 0nM,200nM,1mM,5mM,10mM

- add 1uL of 1M $CaCl_2$ to 100uL of prepared internal solution = 10mM
- prepare a new 100uL internal solution = 0mM
- prepare 9uL 0uM + 1uL 10mM = 1mM
- prepare 5uL 0mM + 5uL 10mM = 5mM
- prepare 49uL 0mM + 1uL 10mM = 200nM

determine from there if more dilutions are necessary

Measuring ΔG/R

- **G** = intensity (PMT output) of CH2 (green channel, calcium indicator Fluo-5) in AFU (14-bit, 0-2^{12}, 0-4096)
- **R** = intensity (PMT output) of CH1 (red channel, Alexa Fluor 594) in AFU (14-bit, 0-2^{12}, 0-4096)
- **ΔG** = change in calcium channel intensity (AFU)
- **ΔG/R** = relative calcium concentration where intensity is adjusted for volume (fractional)
- G_{MAX} = intensity (PMT OUTPUT) of green channel at a saturating calcium concentration (artificially applied)
- R_{MAX} = intensity (PMT OUTPUT) of red channel of the identical image as G_{MAX}
- G_{SAT} = is just the G_{MAX}/R_{MAX} (fractional) at a saturating calcium concentration (artificially applied)
- G/G_{SAT} = a semi-calibrated way to express calcium concentration as a fraction of the saturation concentration
- $\Delta G/G_{SAT}$ = a semi-calibrated way to express a change in calcium concentration as a fraction of the saturation concentration

FINALLY: With an experiment where **G** is measured at two time points (G_1 and G_2)...

$$[Ca2+] \approx \Delta G/G_{SAT} = [(G_2-G_1)/R] / [G_{MAX}/R_{MAX}]$$

Calculating [Ca^{2+}]

Routine Calibration (G_{SAT})

- place a tip-closed glass micropipette slightly above the tissue and image at identical pockel/gain settings.

    doesn't increasing depth require increased intensity?

Extreme Calibration

- We still don't know the reliance of our readings on on pockel setting, gain, gain ratio, or laser power.
- Should we always try to image in the same region of the PMT output? (i.e., basal **G** should be between 500-600 AFU).
- Let's image the same sealed pipette throughout the day, and compare G_{MAX} and R_{MAX}

Procedure

start-up sequence

- turn on the liquid cooling pump
- turn on the switch on the ceiling
- rotate the key on the power supply to the ON position
- once current reaches 21A, press the shutter button
    - the laser will take several minutes to warm up, so we will come back to it
- turn on the GAS (carbogen) and VAC valves
- turn on the power switch behind the computer and turn on the computer
- start bubbling liquid and get it flowing through the chamber
    - ensure the temperature is correctly set
    - set the stimulator current to zero, 'output' switch to off, and turn it on
- mode-lock the laser
    - the oscilloscope should show a pure, unwavering sine wave if mode-locking is to be successful
    - slide the 'B-lock' switch to the middle position briefly and then slide it back to the left
    - if the laser did not mode lock, adjust knobs to achieve clean sine wave and try again
    - if you cannot get it mode locked, consider #re-aligning the laser

optimal sine waveform

mode locked waveform

shut-down sequence

- start running water through the perfusion system
- close the shutter for the laser
- rotate the power supply key to the OFF position
- turn the switch off on the ceiling
- shut down the computer
- turn off the switch behind the computer
- run air through the perfusion system
- turn off the GAS (carbogen) and VAC valves
- turn off the liquid cooling pump

Technique

choosing gain

I still wonder if we can avoid a math headache and avoid PMT nonlinearity issues by adjusting the concentration of A-597 to approximate the intensity of resting intensity of Fluo-5. The PMT can initially be turned high to aid in placing simulators and finding structures, but PMT can then be turned down again for linescan / quantitative imaging.

re-establishing mode lock

When it becomes difficult to mode-lock the laser (often predicted by a small or unclean sine wave), the laser may need to be realigned. Note that the Coherent Mira Optima 900-F Laser Manual is online. It is uncomfortably thorough. This really, really needs to be read and properly documented. For now, the following instructions are from tradition. Also, we could *really* use a concise description of how this laser works, and what all these knobs actually do.

1.) Power-on the laser as described in the #start-up sequence. Do **not** mode-lock the laser.

2.) Familiarize yourself with all of the knobs:

left side	right side	note the continuous wave (CW) and mode lock (ML) positions of the far left switch, as well as the range/reset switch

3.) Maximally open the aperture (slit) by rotating the width adjustment knob clockwise until maximum power is observed (as shown by the number on the far left of the display under the oscilloscope). This is often near 400. "Auto Range" or "Range Reset" may need to be toggled to optimally view power during this adjustment.

4.) Close the aperture (slit) to achieve 50% power by rotating the same knob you just adjusted counter-clockwise until the number on the display is half of maximum. This is often around 200.

5.) Adjust aperture translation for maximum power by rotating the *slit horizontal translation adjustment* knob in either direction to achieve maximum intensity as measured on the device below the oscilloscope.

6.) Adjust the beam pump position by rotating the *pump beam steering* knobs (just the right two) for maximum intensity.

7.) Set the end mirror tilt angle by rotating the *end mirror controls* to maximize power output.

8.) Assess the purity of the sine wave. If it is pure, it will mode-lock easily. If not, go back to step 5. This may need to be done a few times.

ADVANCED TIP

I have found that I get better results if, once mode locked, I slowly decrease laser intensity (by adjusting the iris) back down to 200. As I do this, I often lose mode lock, so I re-establish mode lock, twiddle ALL the knobs to maximize the intensity of the mode-locked peak, then slowly narrow the iris again. After doing this 6-8 times, the laser will mode lock really easily (even though the original sine wave looks surprisingly small, and of the wrong frequency), and the mode-locked laser power will be a steady 200.

re-aligning the laser

Pockel cell

- a magnetic metal disc and the target are required to align it, so request it from the vendor to allow adjustment
- contact service department 608-662-0022 or ryan ryan.falk@bruker-nano.com

laser path

- turn laser on and apply bias to the Pockel cell to allow visualization of the beam
- in prairie view software, tools -> maintenance.
    - It allows opening of shutter and Pockel setting.
    - for all alignment, make sure the galvo position is set to centered
- make sure the beam hits the center of the lower, then upper, tower mirrors.
    - this never needs to be done, unless the optics / mirrors on the table are bumped. It is critical not to bump them. Leave the cover on indefinitely.
- assess the point of the laser on the reference "lens". If it is off center, the ultimate goal is to get it back on center, but it has to be properly centered through the *scan lens*, such that it is exactly centered both with the scan lens in line and out of line with the laser beam path.
- open the top of the tower exposing the galvos and scan lens
- align the laser path through the scan lens
    - ensure the analyzer and polarizer is out of the light path as it can change the beam direction, and you don't use it during scanning
    - with the scan lens IN, adjust the back corner tower-top mirror to center the beam on the objective target. It will look soft. Screws for that are in the back corner and accessed from the top.
    - take the scan lens out (by tilting it forward while removing or inserting it, and setting it in the cavity to the left while it's not in the light path)
    - with the scan lens OUT, adjust the right side mirror to center the beam on the objective target. The beam will look like a tight point of light. Screws for that are facing forward on the right upper region of the tower.
    - repeat several times with the scan lens in line and out of line, until the point of light is centered in both cases.
- screw-down the scan lens by moving the front of the lens to the mark scratched on the floor of the laser path, and tightening it in place.
- ensure the laser beam blocking 'flap' just downstream of the scan lens is fully open before re-applying the lid. Ensure the "open / close" circle on the top of the flap is set to open when it is applied.

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