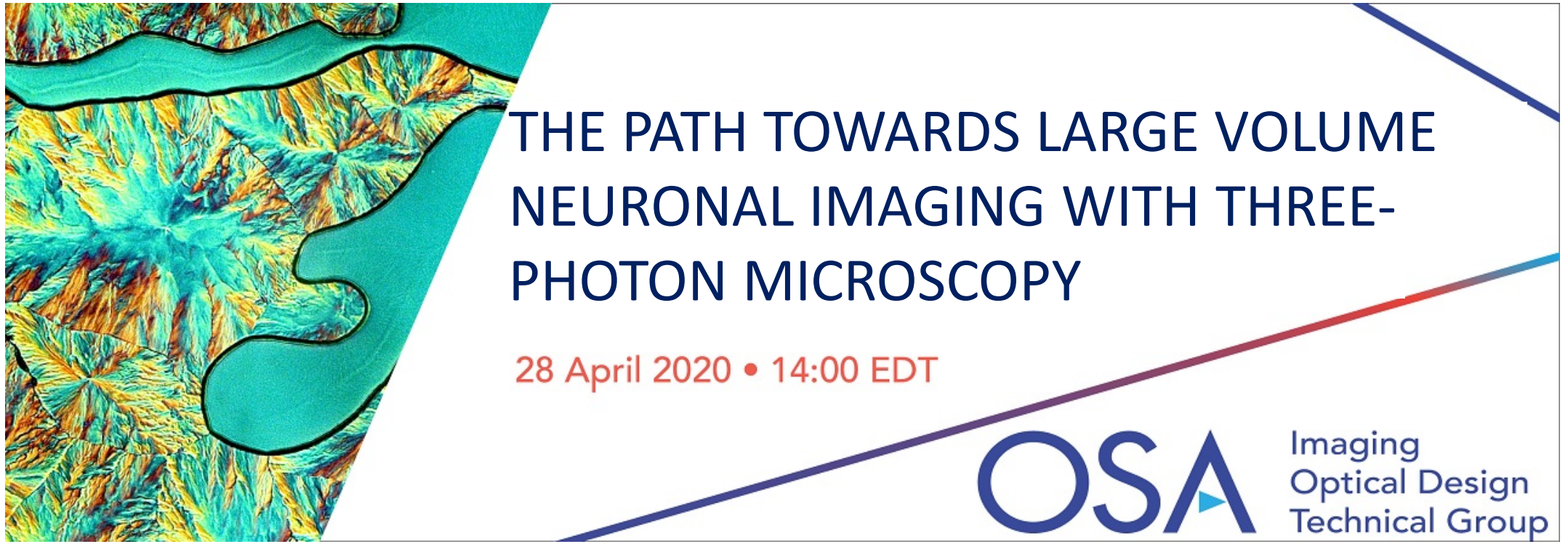

# The Optimization of Three-photon Microscopy for Volumetric Neuronal Imaging

Tianyu Wang, Cornell University

# The OSA Imaging Optical Design Technical Group Welcomes You!



# Technical Group Leadership 2020

Chair
Dr. Maryna L. Meretska

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Dr. Xusan Yang

Social Media Officer
Dr. Marie-Anne Burcklen

Event Officer
Dr. Sarmishtha Satphaty

# Our Technical Group at a Glance

## Our Focus

- “Physics of nonlinear optical materials, processes, devices, & applications”
- 2000 members

## Our Mission

- To benefit *YOU*
- Webinars, social media, publications, technical events, business events, outreach
- Interested in presenting your research? Have ideas for TG events? Contact us at: TGactivities@osa.org.

## Where To Find Us

- Website: https://www.osa.org/en-us/get_involved/technical_groups/fdi/imaging_optical_design_(fd)/
- Facebook: https://www.facebook.com/groups/OSAImagingOpticalDesign/
- LinkedIn: https://www.linkedin.com/groups/8113351/

# Upcoming event

## OSA Imaging Optical Design Technical Group Special Talk at FiO+LS

**When:** Thursday, 17 September, 17:15 - 18:15

**What:** Join the OSA Imaging Optical Design Technical Group for a special talk with **Lyuba Amitonova** of Vrije Universiteit Amsterdam on "Breaking the Abbe and Nyquist Barriers in Far-Field Optical Microscopy". Our featured presenter will give a talk on their research, which will be followed by a moderated question and answer session.

# Today's Webinar

## The Path towards Large Volume Neuronal Imaging with Three-photon Microscopy


### Dr. Tianyu Wang

Applied and Engineering Physics
Cornell University, USA
tw329@cornell.edu


## Speaker's Short Bio:

Ph.D. degree in Applied Physics from Cornell University

Cornell University

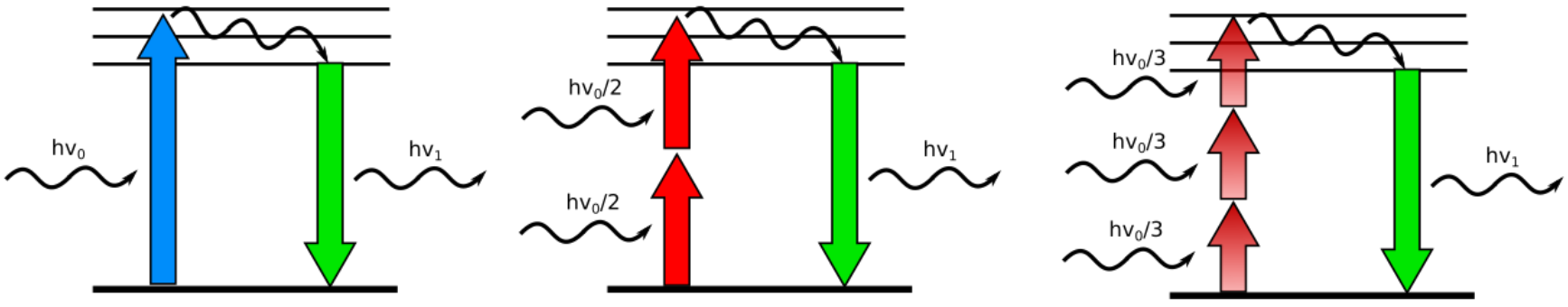
# The Path towards Large-volume Neuronal Imaging with Three-photon Microscopy

Tianyu Wang Ph.D.

Cornell University

- Introduction
- 2PM vs 3PM for Deep Neuronal Imaging
- The Framework for Optimizing 3PM for Large-volume Imaging

# Three-Photon Excitation (3PE) of Fluorescence



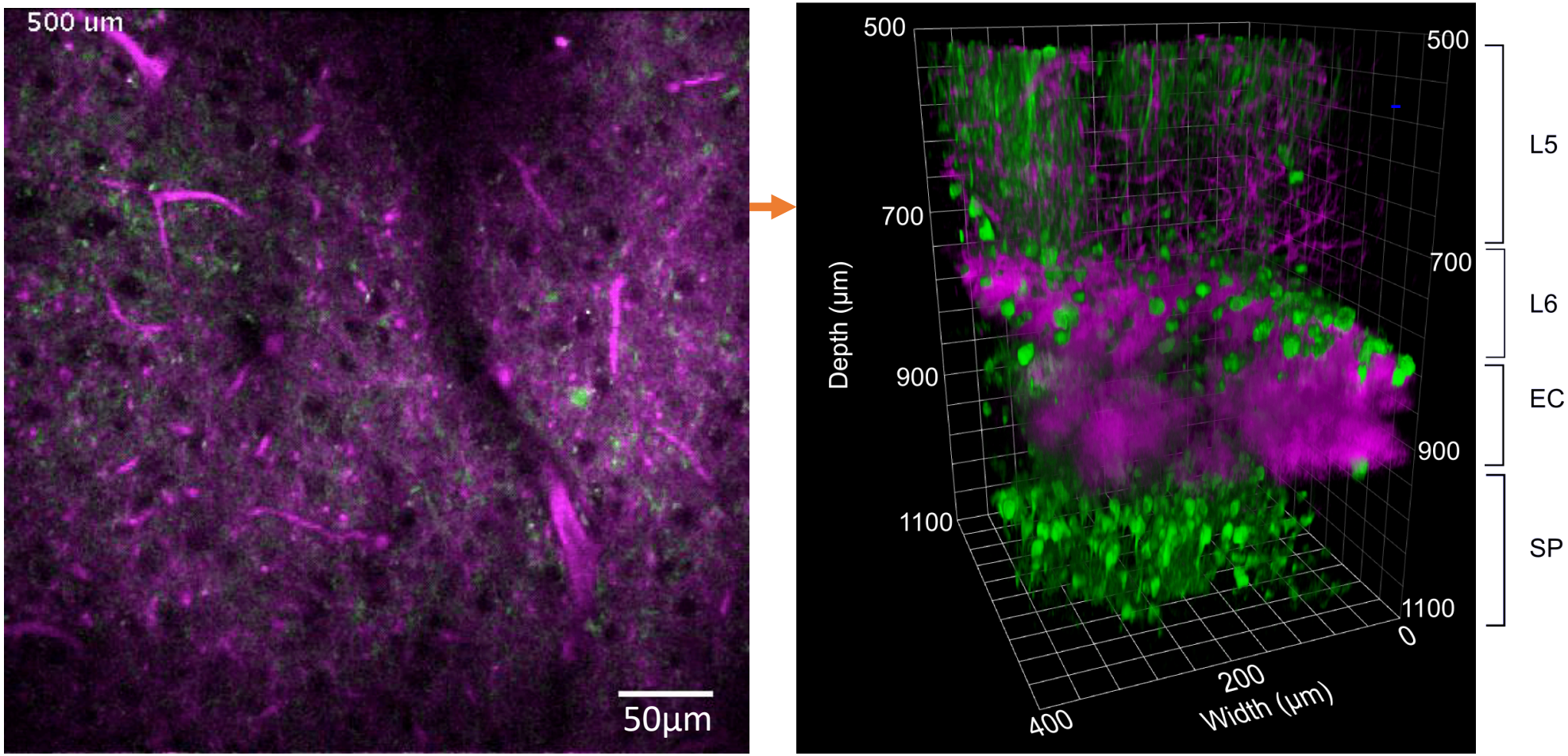

1PE
Signal ∝ $I$

2PE
Signal ∝ $I^2$

3PE
Signal ∝ $I^3$

- 3PE wavelength is around 3 times of one-photon-excitation wavelength => longer wavelength in infrared
- 3PE has smaller cross section than two-photon excitation (2PE) => ~10x stronger excitation intensity is needed as 2PE

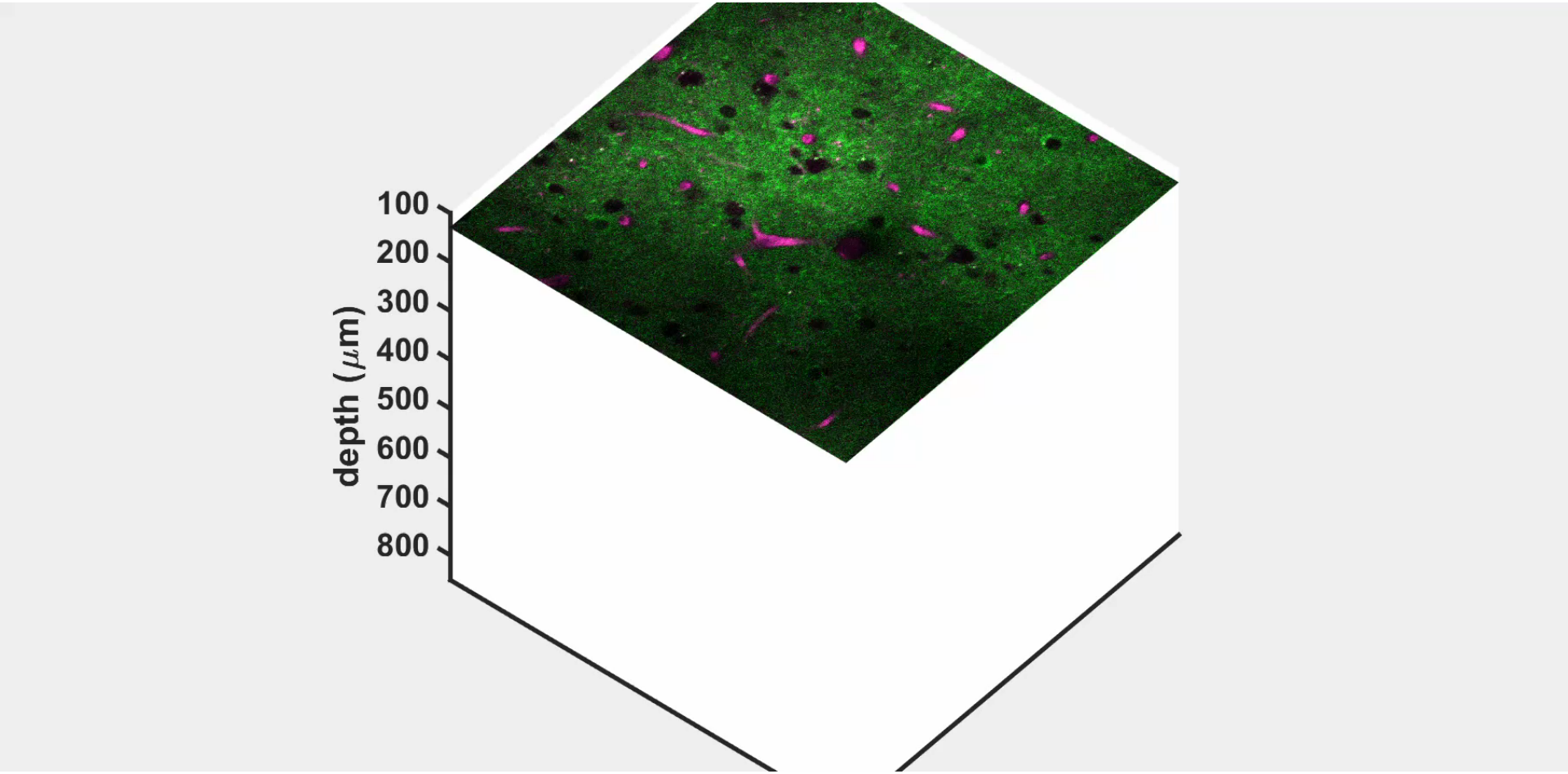
# Neuronal Imaging in Deeper Brain by 1300-nm Three-photon Microscopy (3PM)




"*In vivo* three-photon imaging of activity of GCaMP6-labeled neurons deep in intact mouse brain" *Nat. Methods* (Ouzounov, T. Wang, …, C. Xu, 2017) https://doi.org/10.1038/nmeth.4183

Green: Fluorescence from GCaMP6 calcium indicator
Magenta: Third Harmonic Generation (THG)

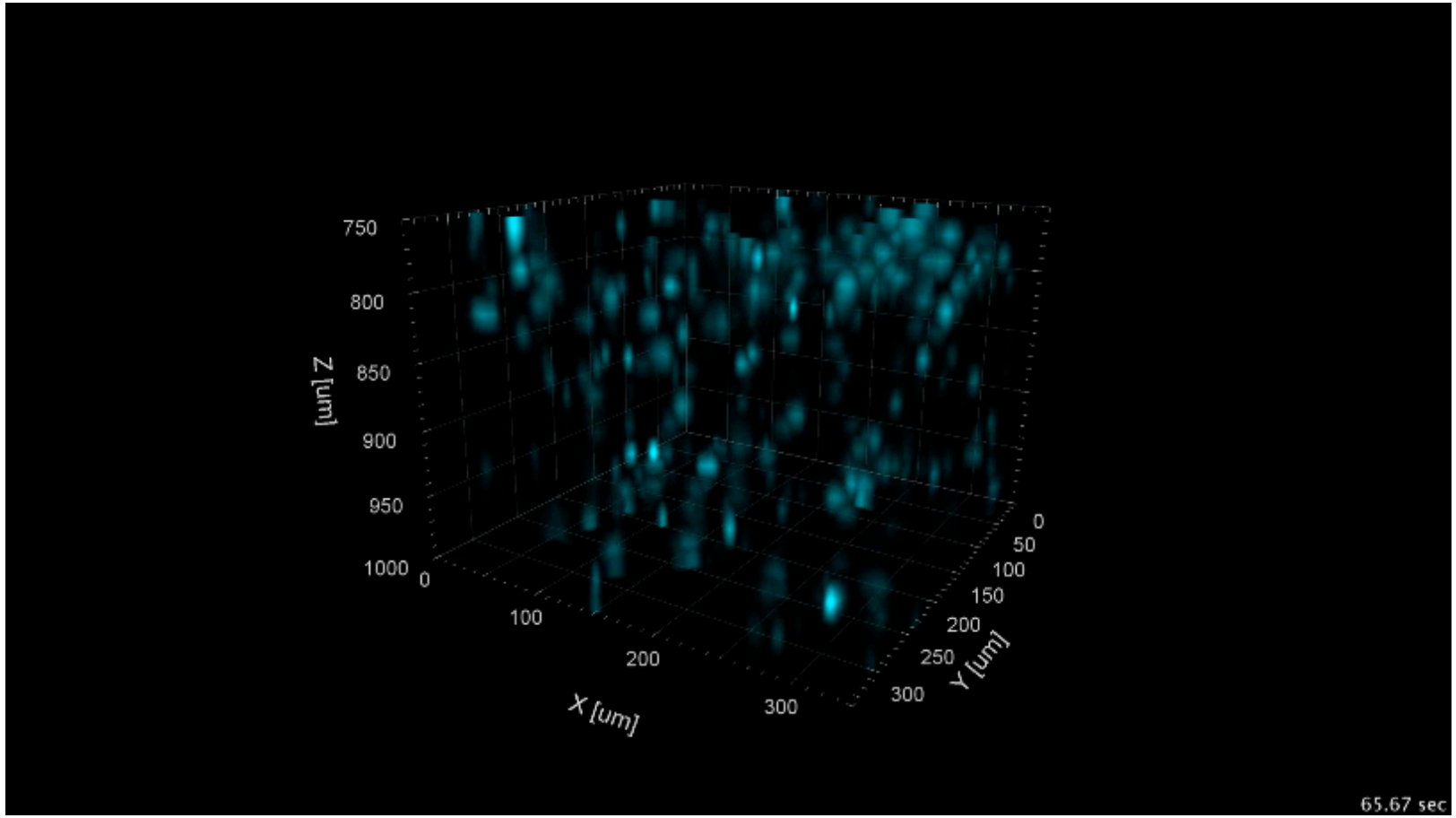
# Somatosensory Cortex in Densely Labeled Brain: L1 to L6




FOV: 250x250 μm; Frame Rate: 8.49Hz  Transgenic mouse CamKII/tTA-GCaMP6s (20 weeks)
Green: Fluorescence
Magenta: THG

Thanks to Jacob Reimer from Baylor College of Medicine for the animation.

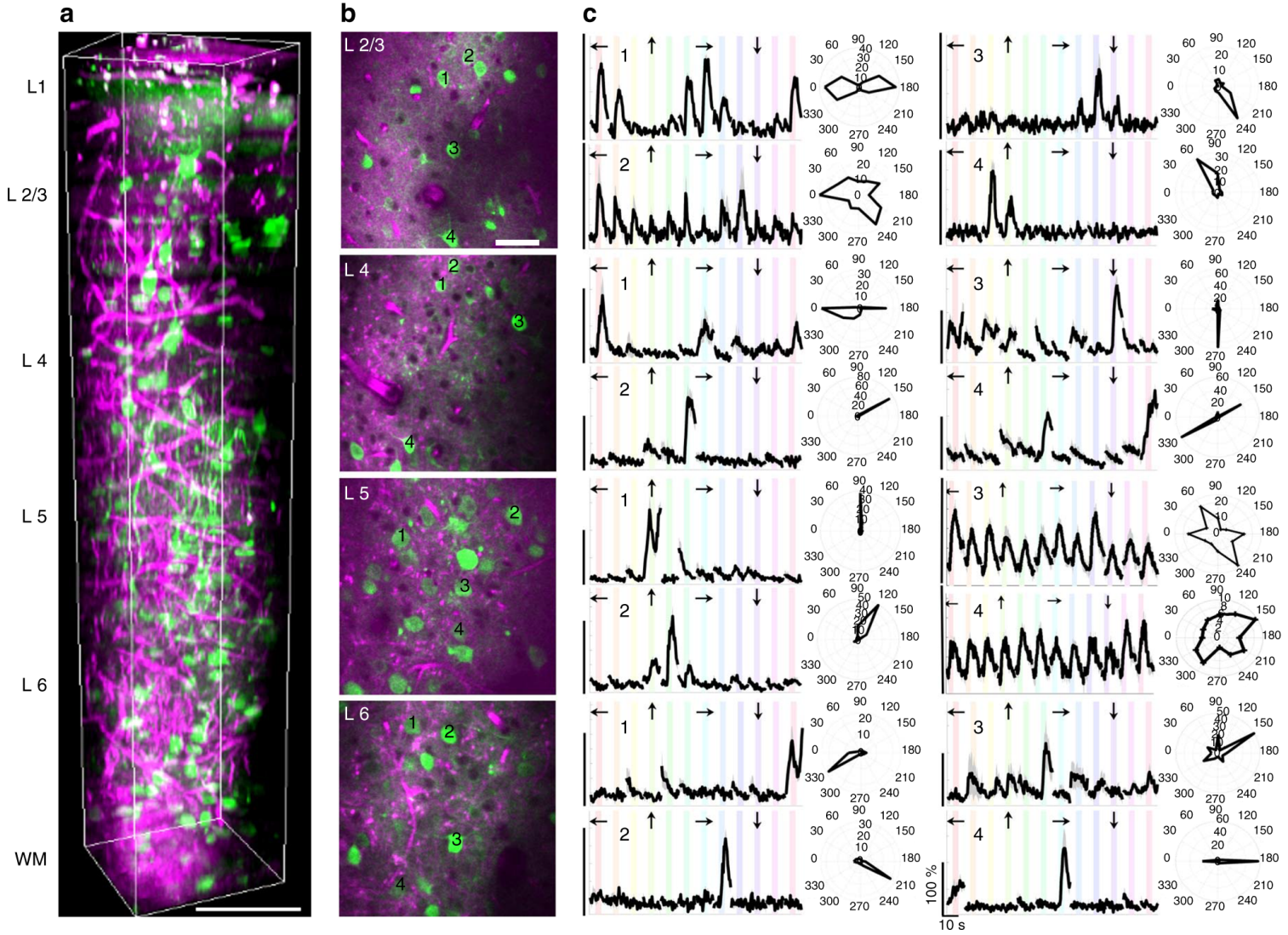
# Volumetric Ca $^{2+}$ Imaging in the Deep Cortex of Mouse Brain




3D rendering of a 5-min volumetric 3p recording in mouse PPC L6 through intact cortex at 750-1,000 µm depth, 340 × 340 × 250 µm FOV, 3.9 Hz, cytosolic GCaMP6f. Playback speed: 5×

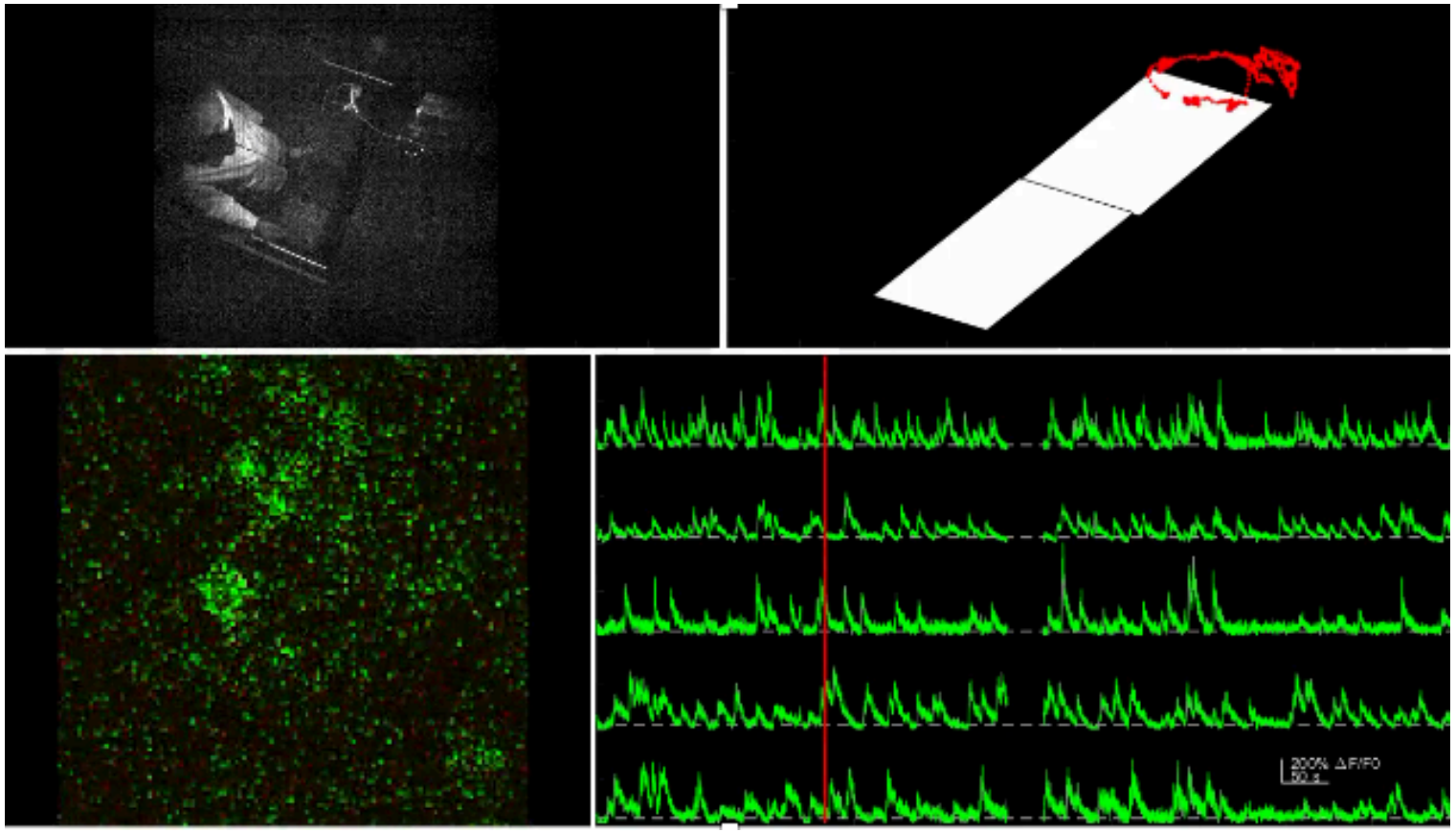
"Volumetric $Ca^{2+}$ Imaging in the Mouse Brain Using Hybrid Multiplexed Sculpted Light Microscopy" *Cell* (Weinsenburger …, Vaziri, 2019) https://doi.org/10.1016/j.cell.2019.03.011

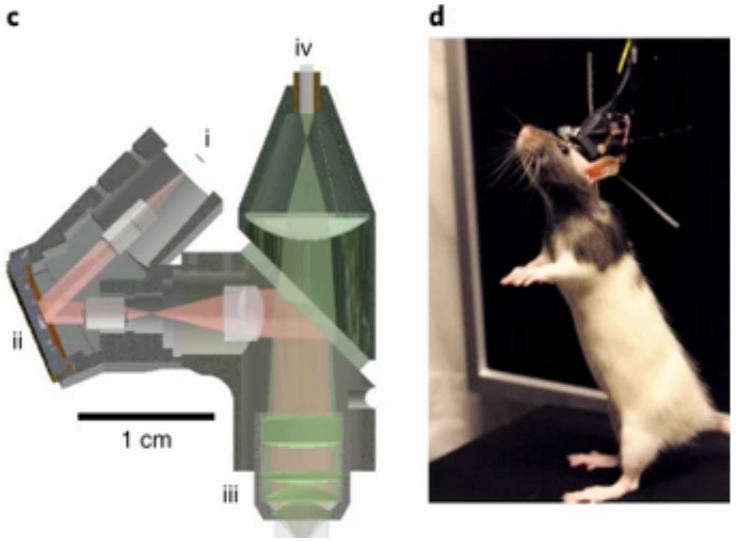
# Visual Responses at All Layers in V1




"Functional imaging of visual cortical layers and subplate in awake mice with optimized three-photon microscopy
" *Nat. Comm.* (Yildirim, .. P. T. C. So, and M. Sur 2019) https://doi.org/10.1038/s41467-018-08179-6

# Head mounted 1300-nm 3PM in Freely Moving Rats



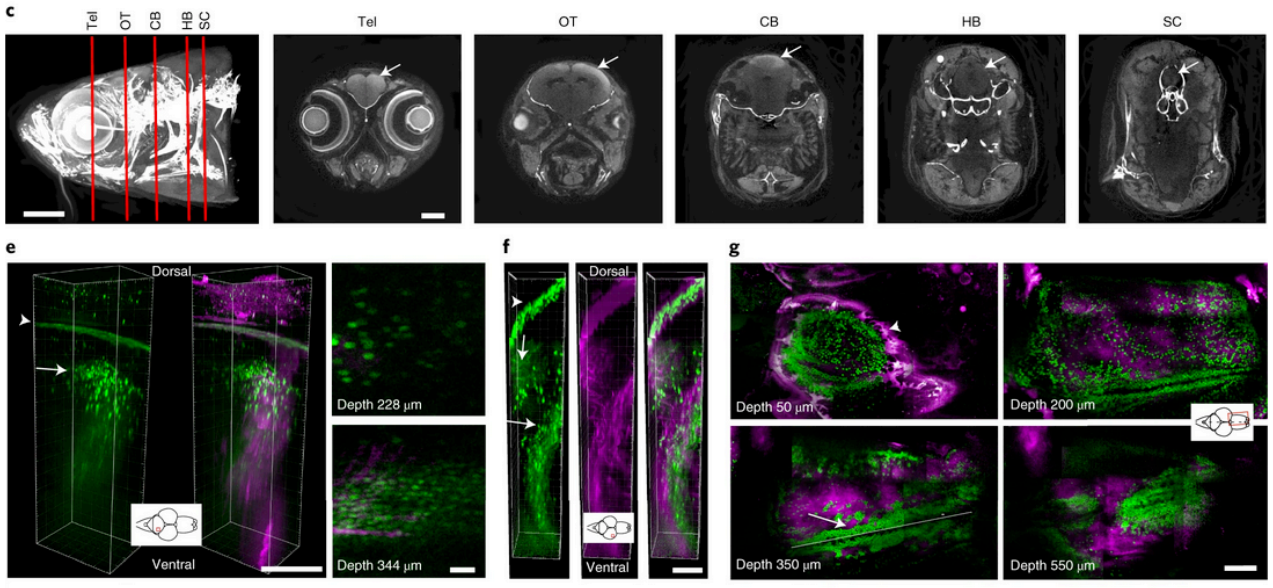



"Three-photon head-mounted microscope for imaging deep cortical layers in freely moving rats" *Nat. Methods* (Klioutchnikov …, Kerr, 2020) https://doi.org/10.1038/s41592-020-0817-9

Imaging at ~1mm in posterior parietal cortex of freely moving rat for ~3.6 min.

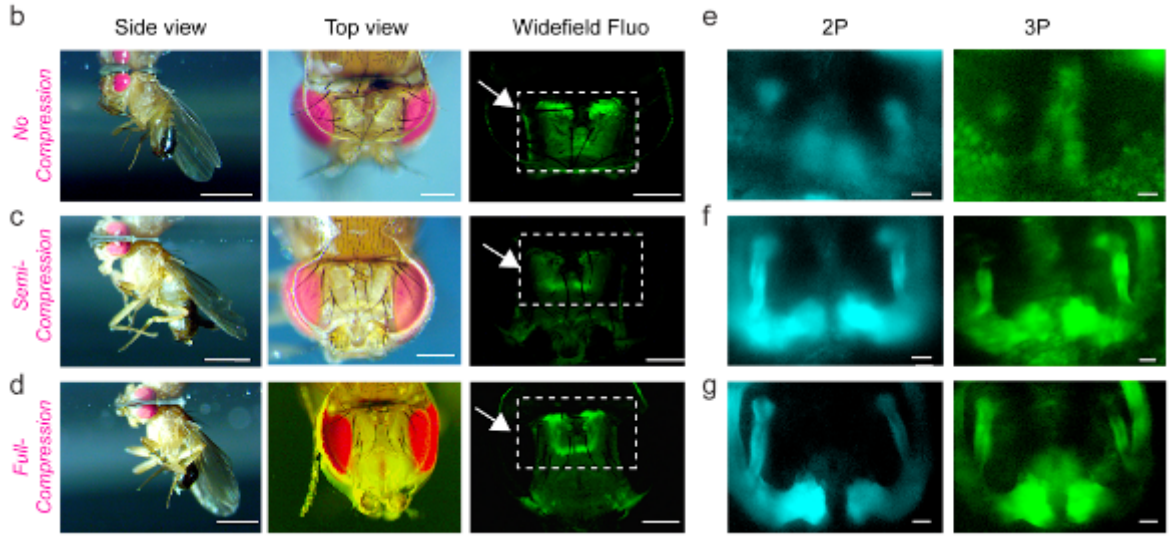
# 3P Neuronal Imaging in Non-rodents

**zebrafish**




"Deep three-photon imaging of the brain in intact adult zebrafish" *Nat. Methods* (D. Chow, …, C. Xu, & J. Fetcho, 2020) https://doi.org/10.1038/s41592-020-0819-7

**fruitfly**

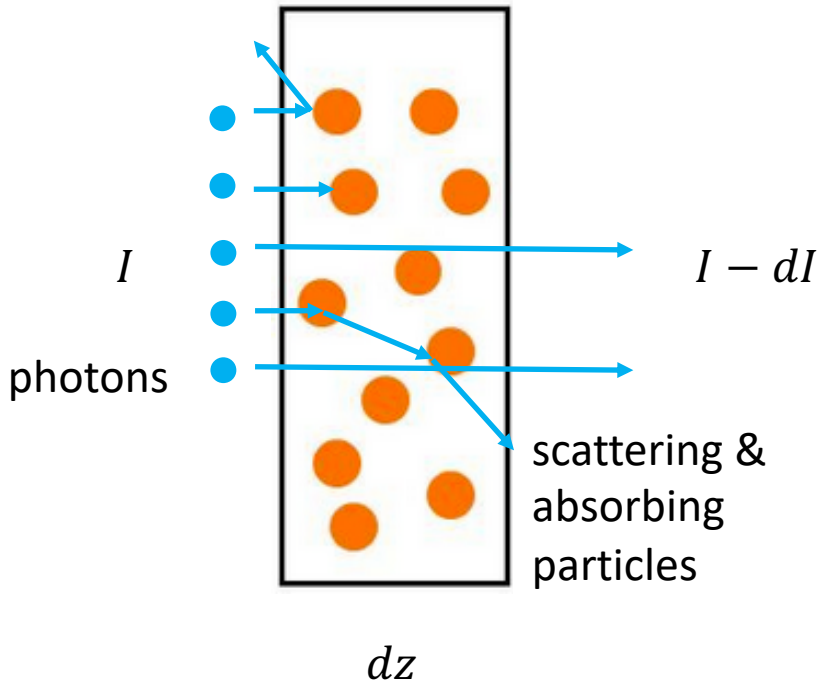



"Non-invasive multiphoton imaging of neural structure and activity in *Drosophila*" *BioRxiv* (Aragon, M. Wang, …, Yapici 2019) https://doi.org/10.1101/798686

- Introduction
- 2PM vs 3PM for Deep Neuronal Imaging
- The Framework for Optimizing 3PM for Large-volume Imaging

# Excitation light attenuates exponentially in scattering and absorbing media

- Ballistic photons: un-scattered excitation light that contributes to MPE signal at the focus.
- Tissue attenuation of ballistic photons = scattering + absorption
- Excitation power at the focus $\propto \exp(-z/\mathrm{EAL})$, where $\mathrm{EAL}$ is the effective attenuation length that takes account of both scattering and absorption.




The number of photons absorbed or scattered is both proportional to the total number of photons

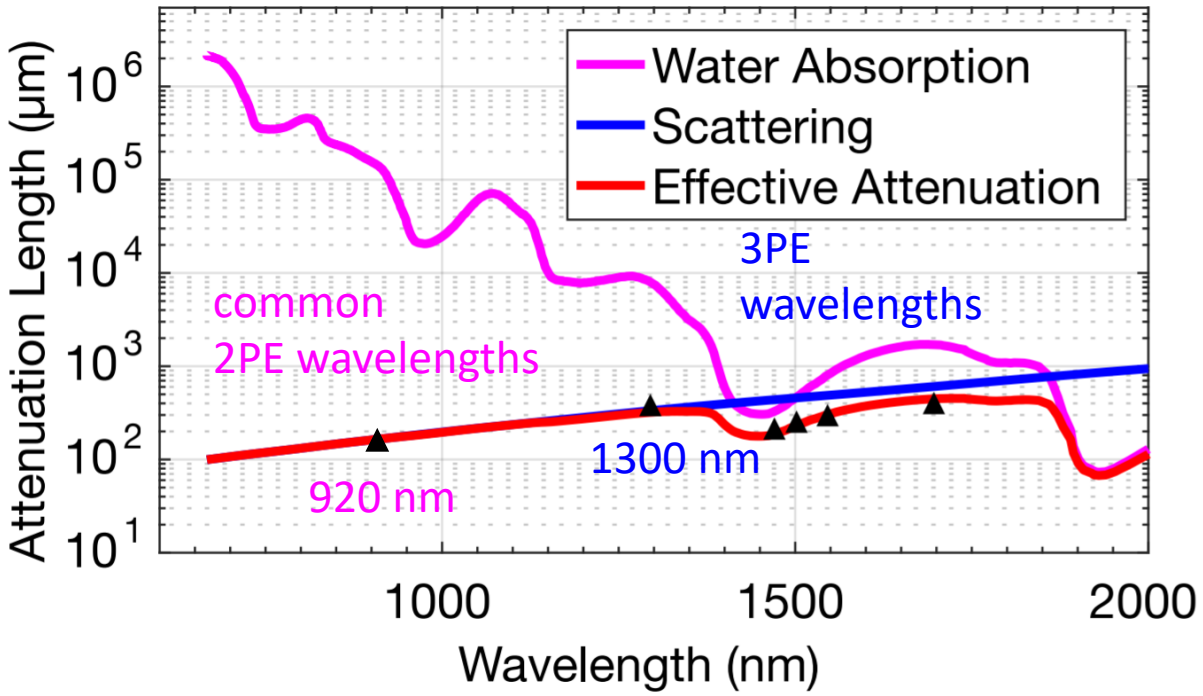
$$\frac{dI}{dz} \propto -I \quad \Rightarrow \quad I(z) = e^{-z/EAL}$$

Excitation power grows exponentially with depth!

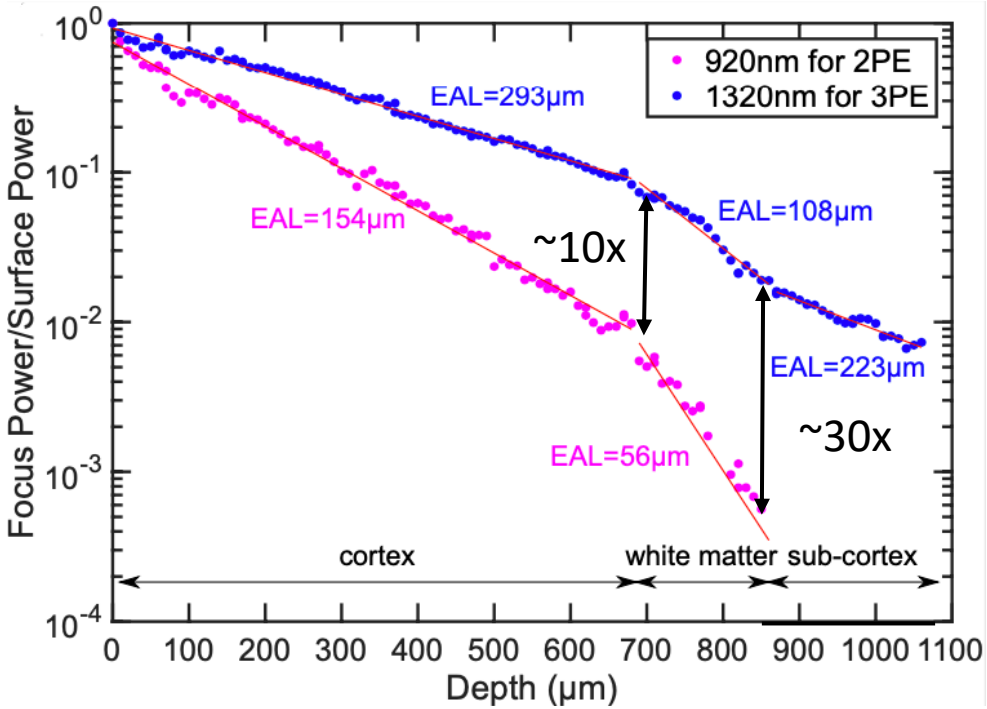
# Long-wavelength excitation experiences less attenuation

3PE wavelength is longer than 2pe for the same fluorophore, and experiences :

- less scattering (longer scattering length)
- more absorption (shorter absorption length)
- overall less attenuation (longer attenuation length)




"Comparing the effective attenuation lengths for long wavelength in vivo imaging of the mouse brain" *Biomed. Opt. Express* (M. Wang …, C. Xu, 2020) https://doi.org/10.1364/BOE.9.003534

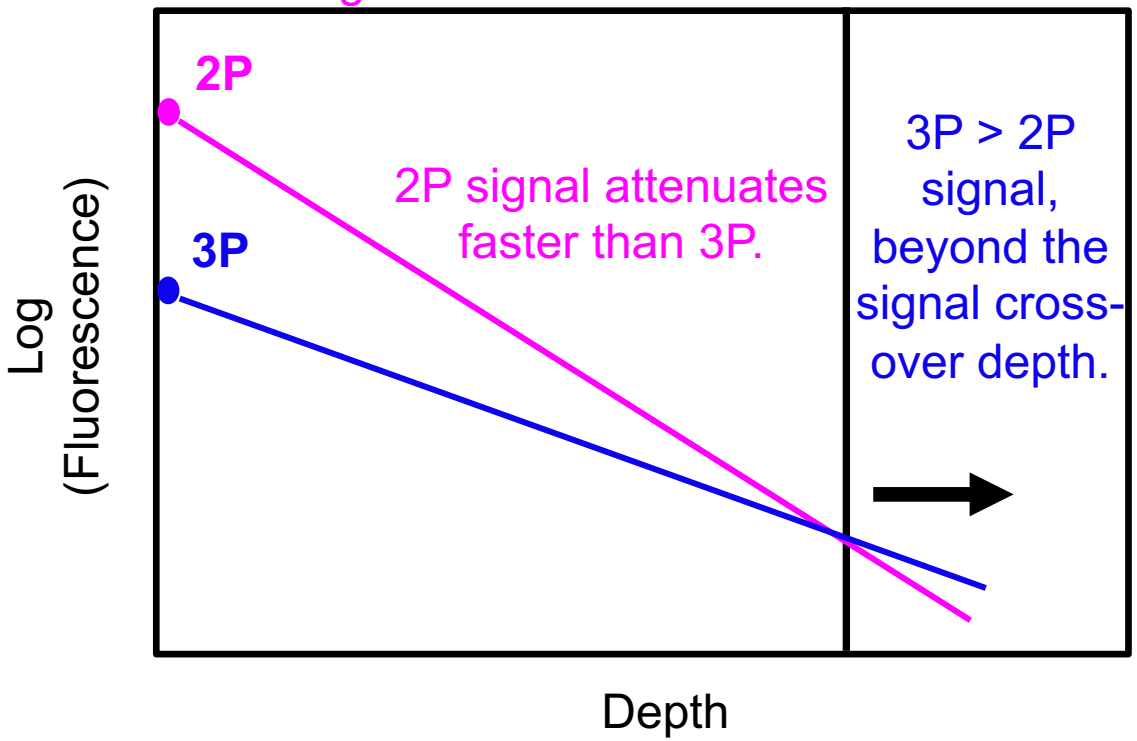


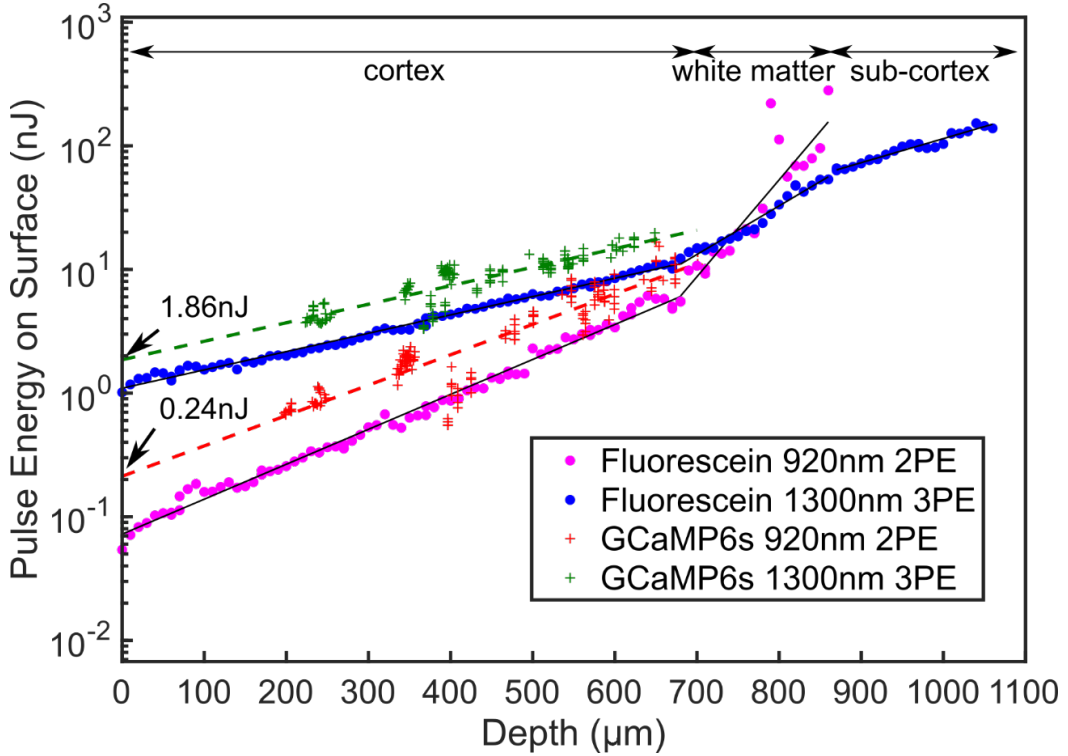

"Quantitative analysis of 1300-nm three-photon calcium imaging in the mouse brain" *eLife* (T. Wang, C. Wu, …, C. Xu, 2020) https://doi.org/10.7554/eLife.53205

# Signal Crossover between 2P and 3P Signals

2PE and 3PE signal vs imaging depth given the same excitation pulse energy on the sample surface

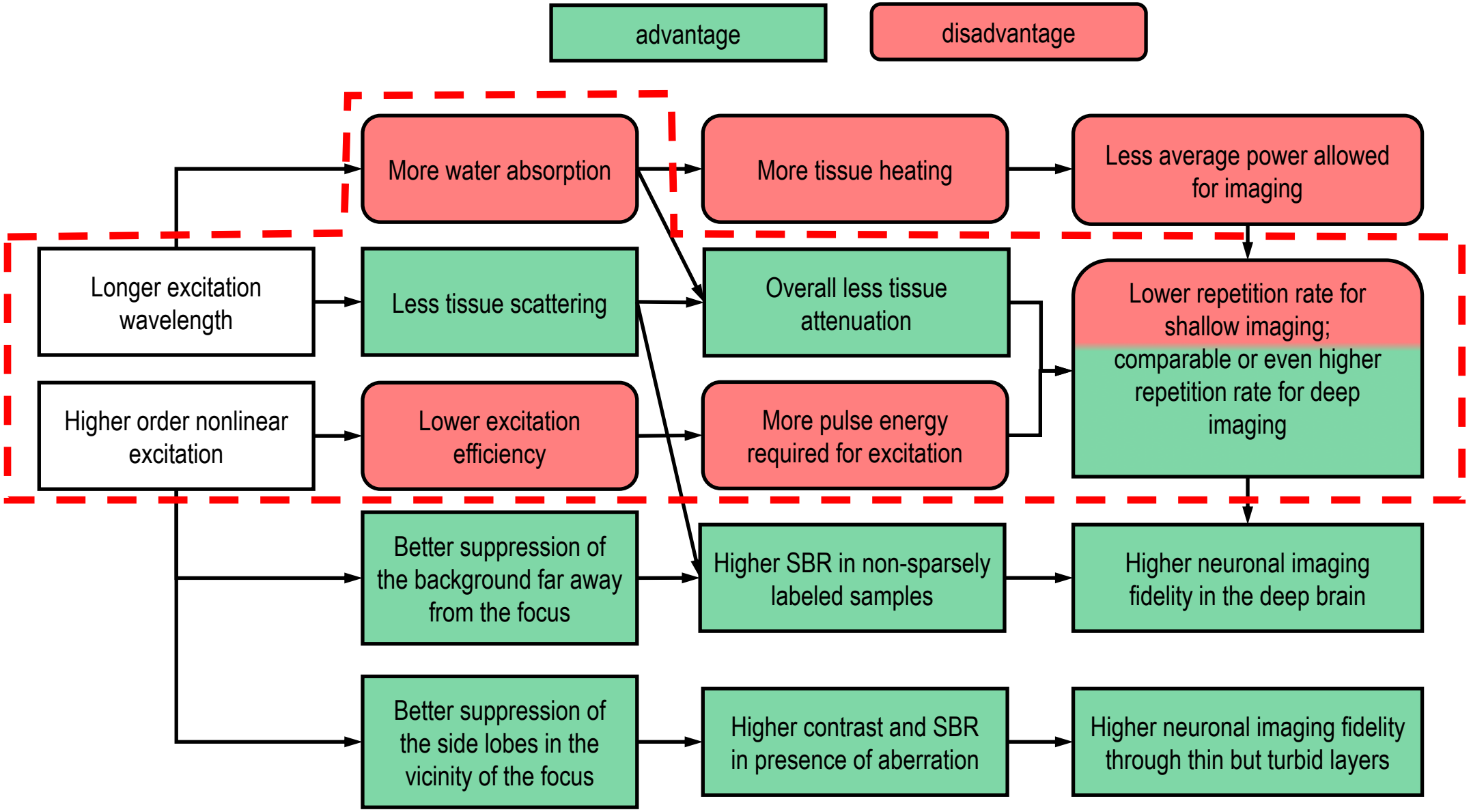
2P > 3P signal, since 2P cross section is larger.



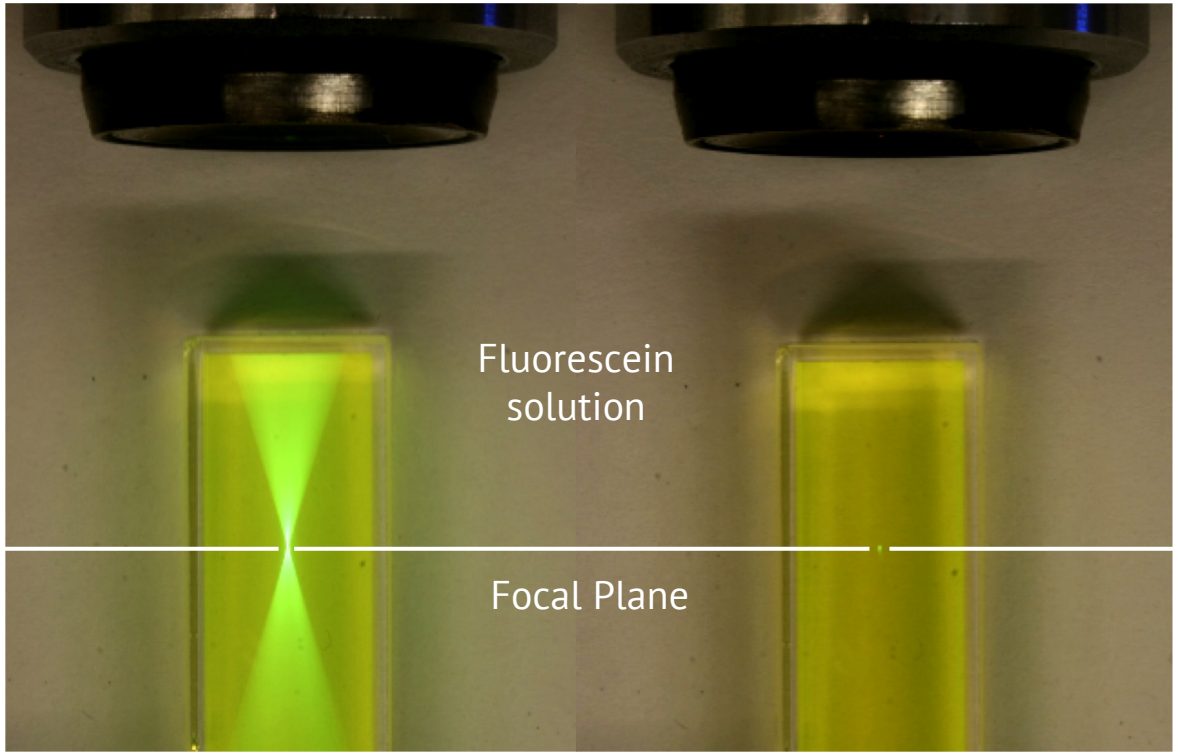

The cross-over is measured where 2P and 3P have equal signals with equal excitation pulse energies on the brain surface.

"Three-photon neuronal imaging in deep mouse brain " *Optica* (T. Wang and C. Xu, 2020) https://doi.org/10.1364/OPTICA.395825


advantage
disadvantage
More water absorption
More tissue heating
Less average power allowed for imaging
Longer excitation wavelength
Less tissue scattering
Overall less tissue attenuation
Lower repetition rate for shallow imaging; comparable or even higher repetition rate for deep imaging
Higher order nonlinear excitation
Lower excitation efficiency
More pulse energy required for excitation
Better suppression of the background far away from the focus
Higher SBR in non-sparsely labeled samples
Higher neuronal imaging fidelity in the deep brain
Better suppression of the side lobes in the vicinity of the focus
Higher contrast and SBR in presence of aberration
Higher neuronal imaging fidelity through thin but turbid layers

# 1P vs 2P Background in Uniformly Labeled Samples

$1PE \propto Intensity$ $2PE \propto Intensity^2$

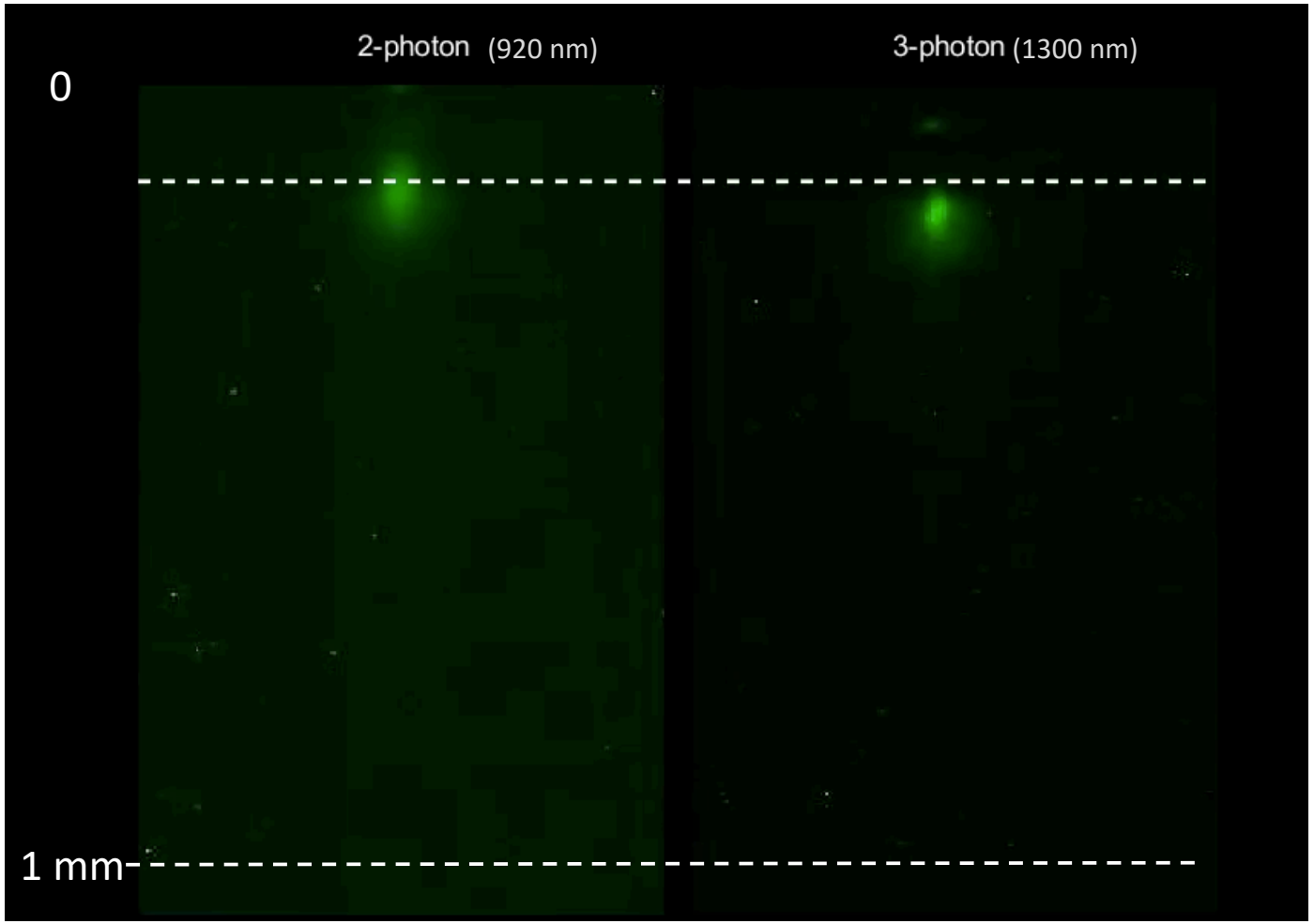



- 1PE excites fluorescence in the entire labeled &illuminated volume.
- 2PE fluorescence is more concentrated at the focus, where the intensity is the highest.
- 2PM is capable of forming 3D image like confocal microscopy, without the help of the confocal pinhole.
- However, 2PE still generates background…

# 2P vs 3P Background in Scattering Samples

**Sideview of fluorescence distribution in scattering tissue phantom**

$2PE \propto Intensity^2$ $3PE \propto Intensity^3$

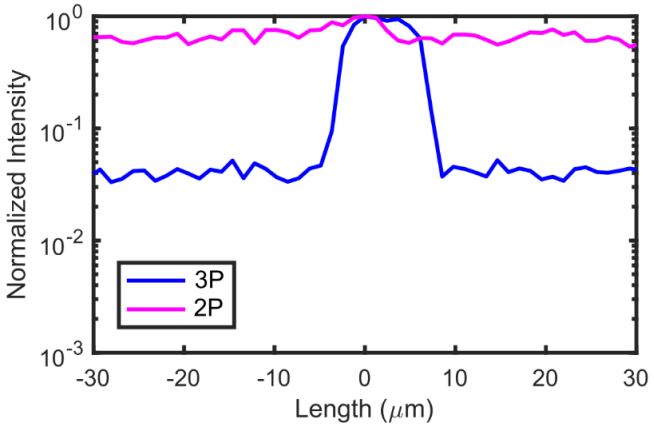



- In *scattering and absorbing* samples, the imaging power increases exponentially with depth due to sample attenuation.
- The increase in 2P background results from the high power deposited on the illuminated volume.
- For deep imaging in *uniformly labeled* samples, 2P out-of-focus background can be comparable to the 2P signal at the focus.
- 3P background is ignorable at the same depth, when exciting the same fluorophore.

"Three-photon head-mounted microscope for imaging deep cortical layers in freely moving rats" (Klioutchnikov …, Kerr, 2020) https://doi.org/10.1038/s41592-020-0817-9

# 2P vs 3P Background: *in vivo* Imaging

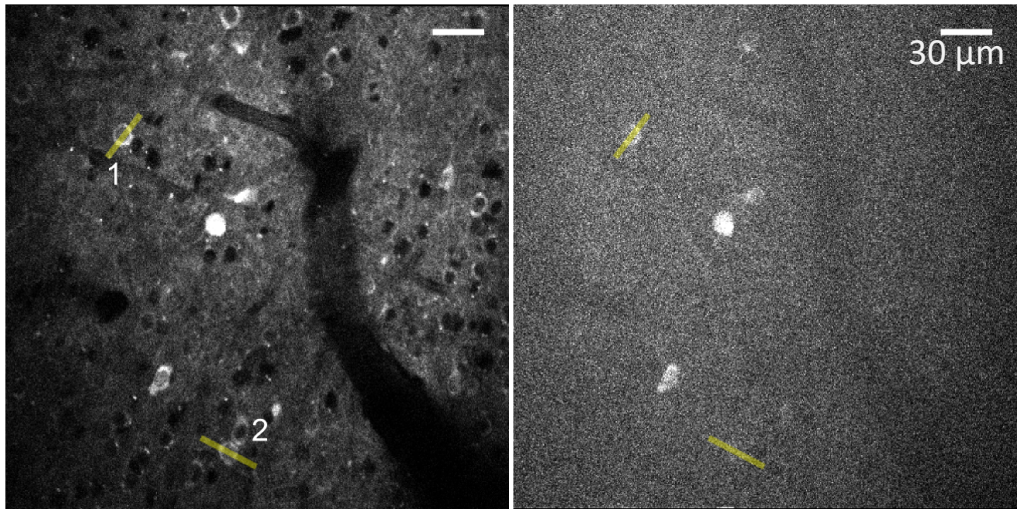
- In uniformly labeled samples, 2PM imaging depth is fundamental limited by the out-of-focus fluorescence background.
- The background reduces *image contrast*, and introduces additional *noise*.




The intensity profile along a line across the same blood vessel imaged by 2PM and 3PM.

**Transgenic mouse with GCaMP6s at 780 µm**

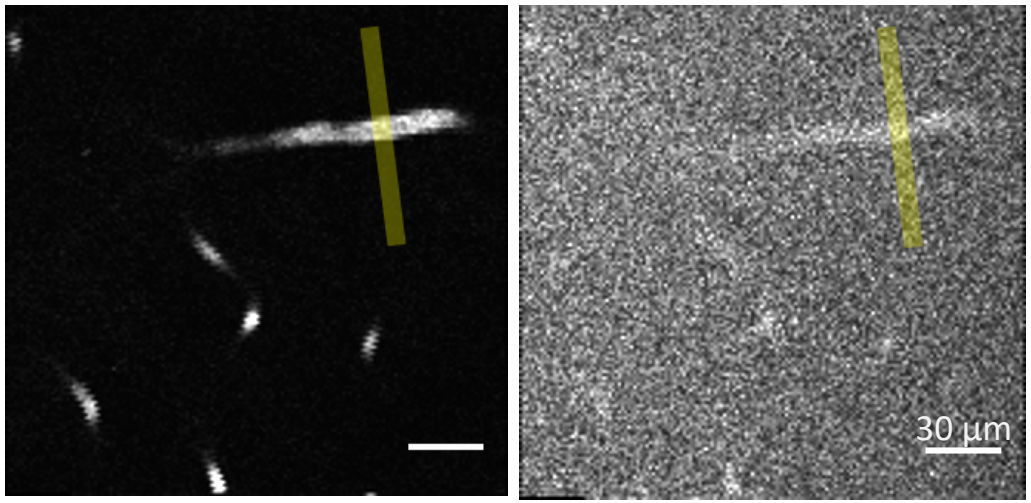
1300 nm 3PM | 920 nm 2PM



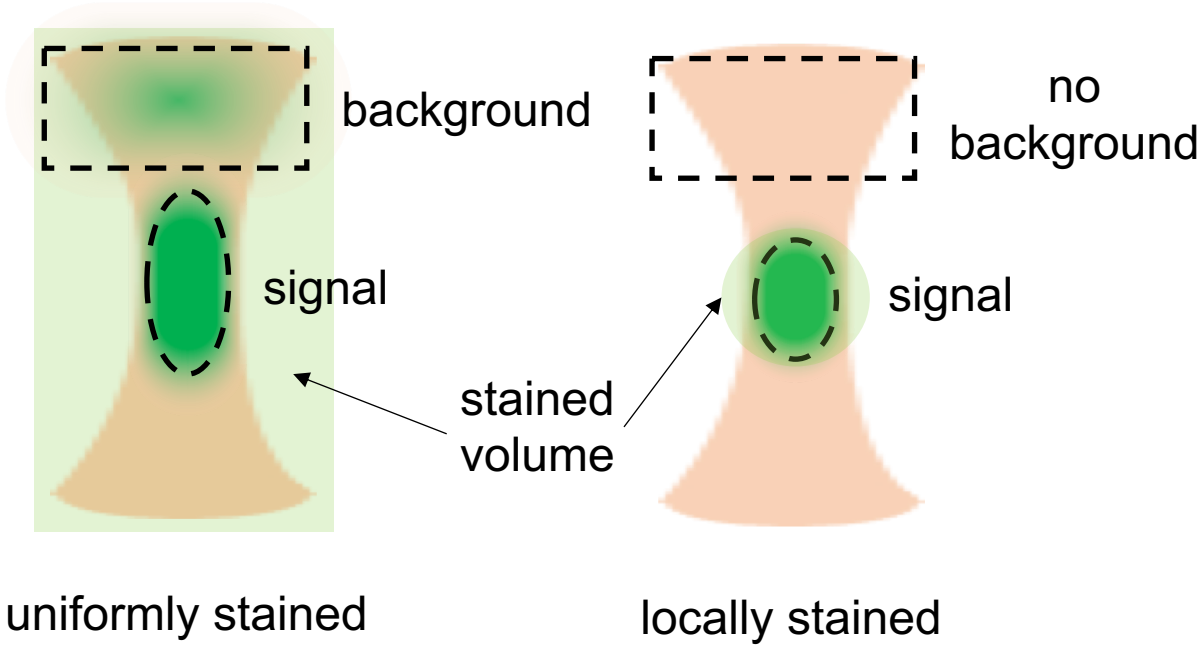

"*In vivo* three-photon imaging of activity of GCaMP6-labeled neurons deep in intact mouse brain" *Nat. Methods* (Ouzounov, T. Wang, …, C. Xu, 2017) https://doi.org/10.1038/nmeth.4183

**Fluorescein-labeled blood vessels at 790 µm**

1300 nm 3PM | 920 nm 2PM



# Signal-to-Background Ratio




- **signal** = fluorescence generated by a diffraction-limited focus* with the same power as the actual power at the focus.
- **background** = total fluorescence - signal
- A common figure of merit is **signal-to-background ratio** (SBR):

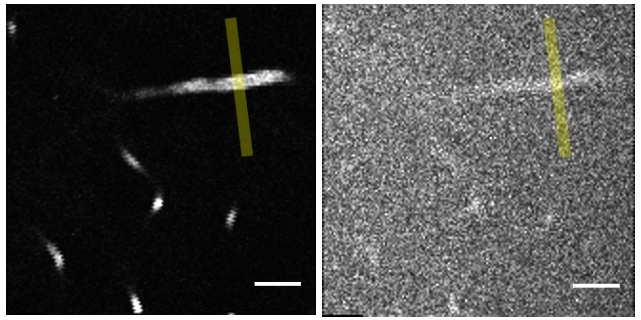
$$\mathrm{SBR} = \frac{\mathrm{signal}}{\mathrm{background}}$$

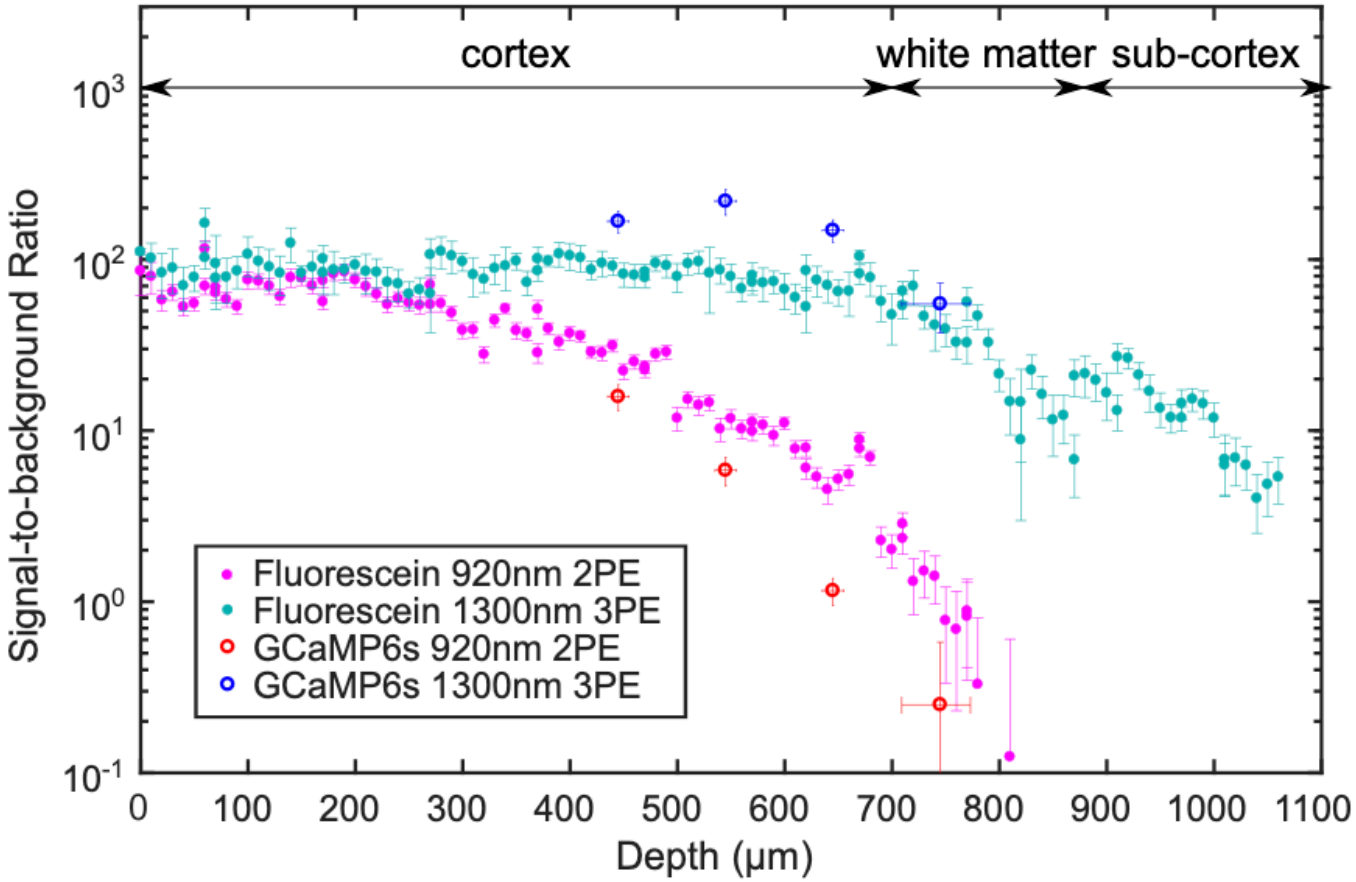
- SBR is practically measured by taking the ratio of the intensity of stained over unstained feature in an image.

# 3P has vastly reduced background in uniformly labeled mouse brain

1300 nm
3PM

920 nm
2PM





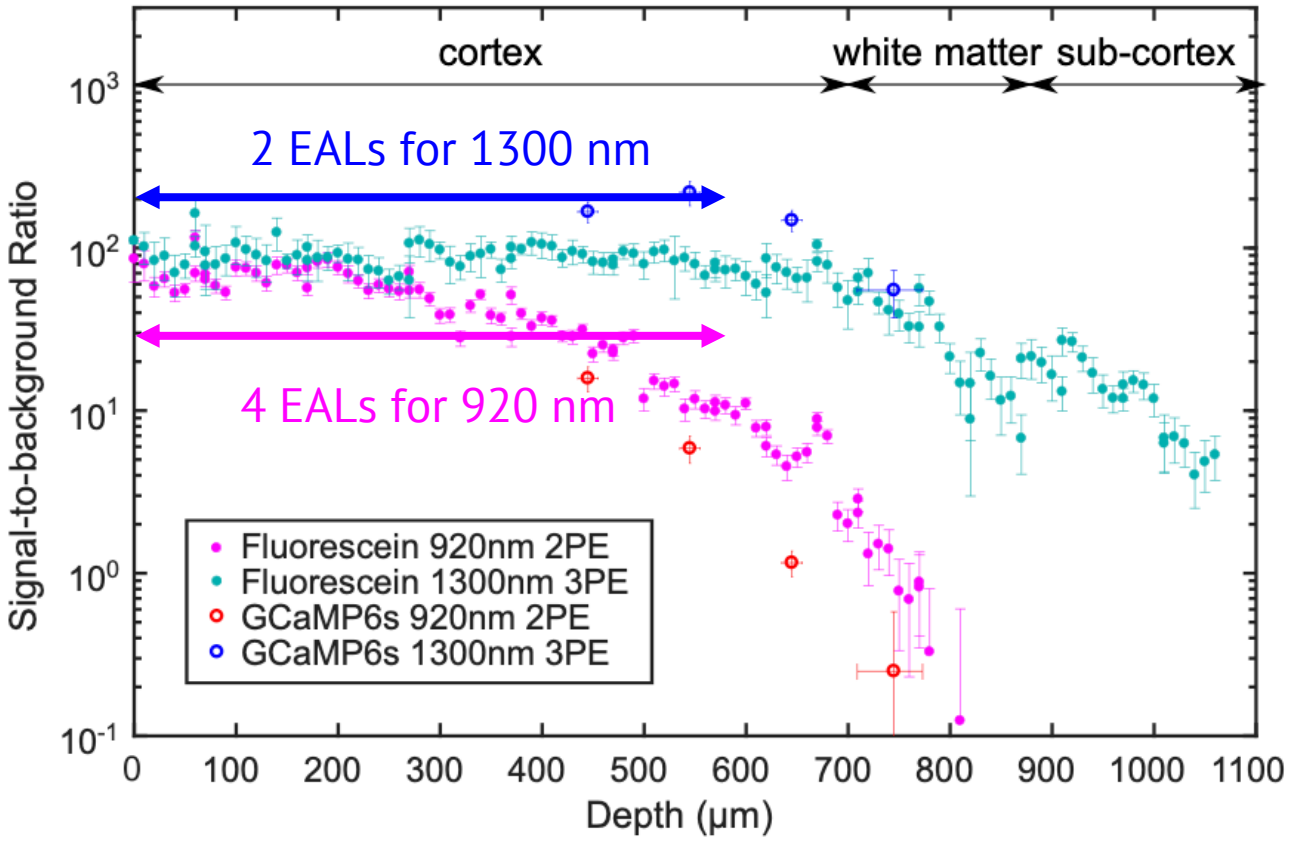

For both labeled blood vessels and neurons:
2P SBR drops below 1 between 700 and 800 μm
3P SBR remains above 20 at 1060 μm, below the white matter.

"Quantitative analysis of 1300-nm three-photon calcium imaging in the mouse brain" *eLife* (T. Wang, C. Wu, …, C. Xu, 2020) https://doi.org/10.7554/eLife.53205

# Longer attenuation length reduces background

MPM background fluorescence depends:

- strongly on the normalized imaging depth $z/\mathrm{EAL}$. Since excitation power $\propto \exp(z/\mathrm{EAL})$, and background scales with imaging power in a polynomial fashion.
- weakly on the absolute value of $\mathrm{EAL}$ with approximately logarithmic relation *.

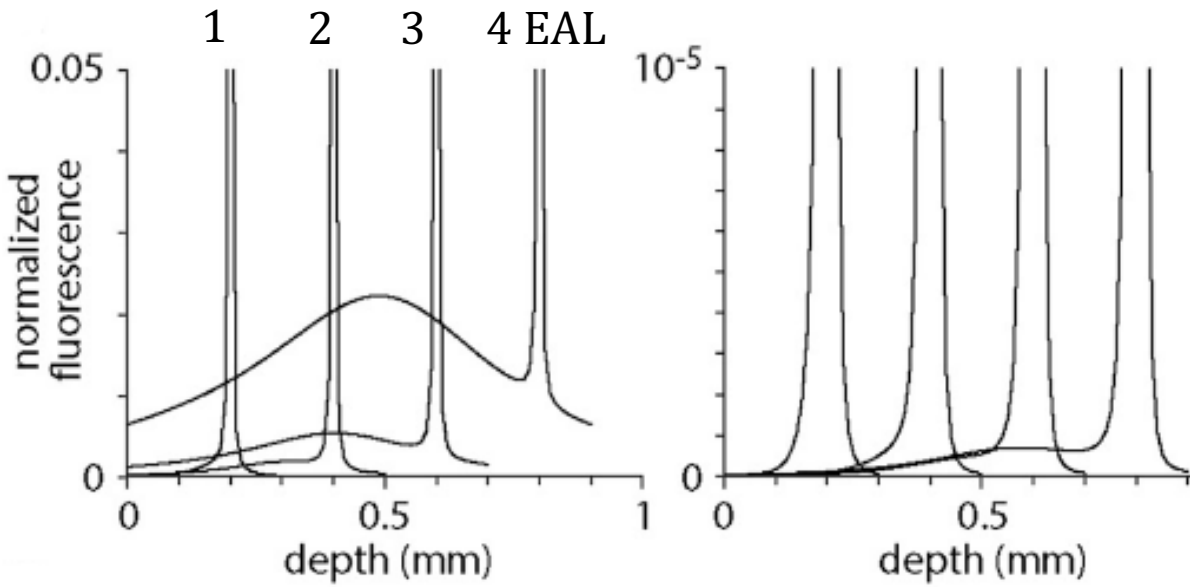



A longer $\mathrm{EAL}$ allows 3P to the number of EALs, and therefore background at the same physical imaging depth as 2P!

*"Maximum imaging depth of two-photon autofluorescence microscopy" (Durr..., Ben-Yakar, 2011) https://doi.org/10.1117/1.3548646

# 2P vs 3P Background at the same z/EAL: Numerical Studies

**Calculation from analytical expressions of Gaussian beam and scattering diffusion**

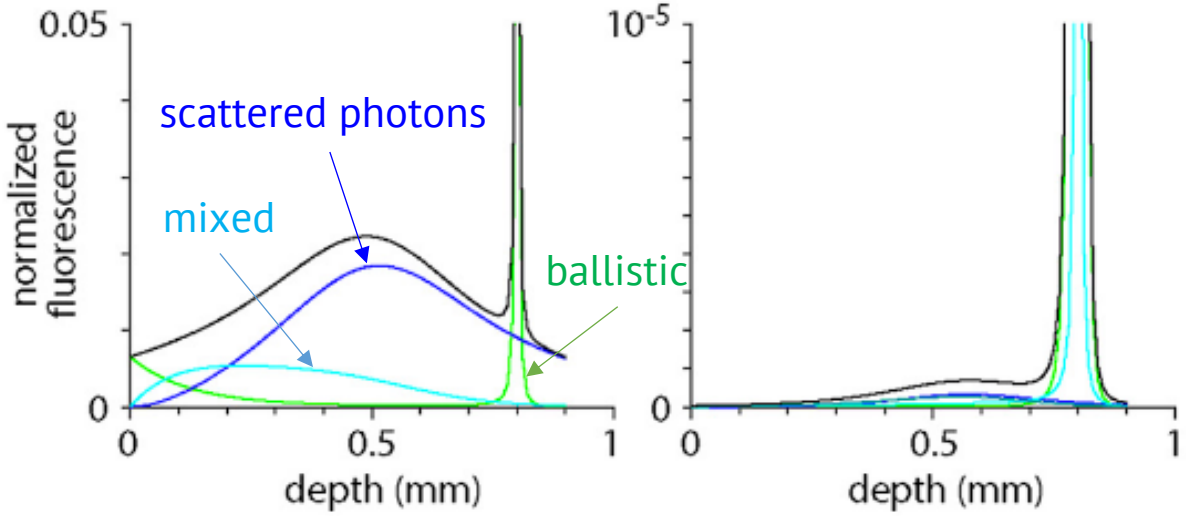



2PE 3PE

$\mathrm{EAL} = 200\ \mu\mathrm{m}$

"Superficial Bound of the Depth Limit of Two-Photon Imaging in Mouse Brain" *eNeuro* (Takasaki..., Waters, 2020) https://doi.org/10.1523/ENEURO.0255-19.2019

**Sources of fluorescent background**

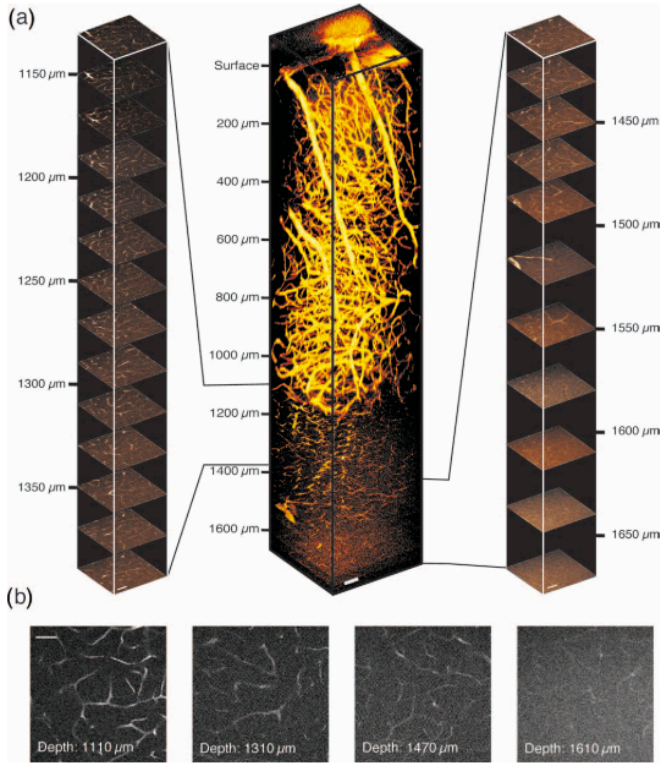


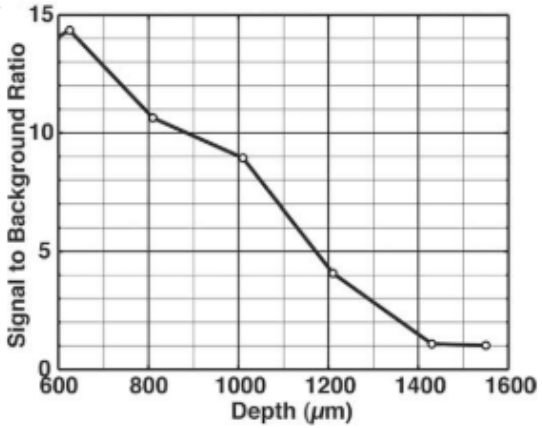

2PE 3PE

Even with the same $z/\mathrm{EAL}$, 3P still produces less background, since $I^3$ dependence significantly reduces the chance of out-of-focus background generation!

# 2P vs 3P Background at the same z/EAL: Experiments

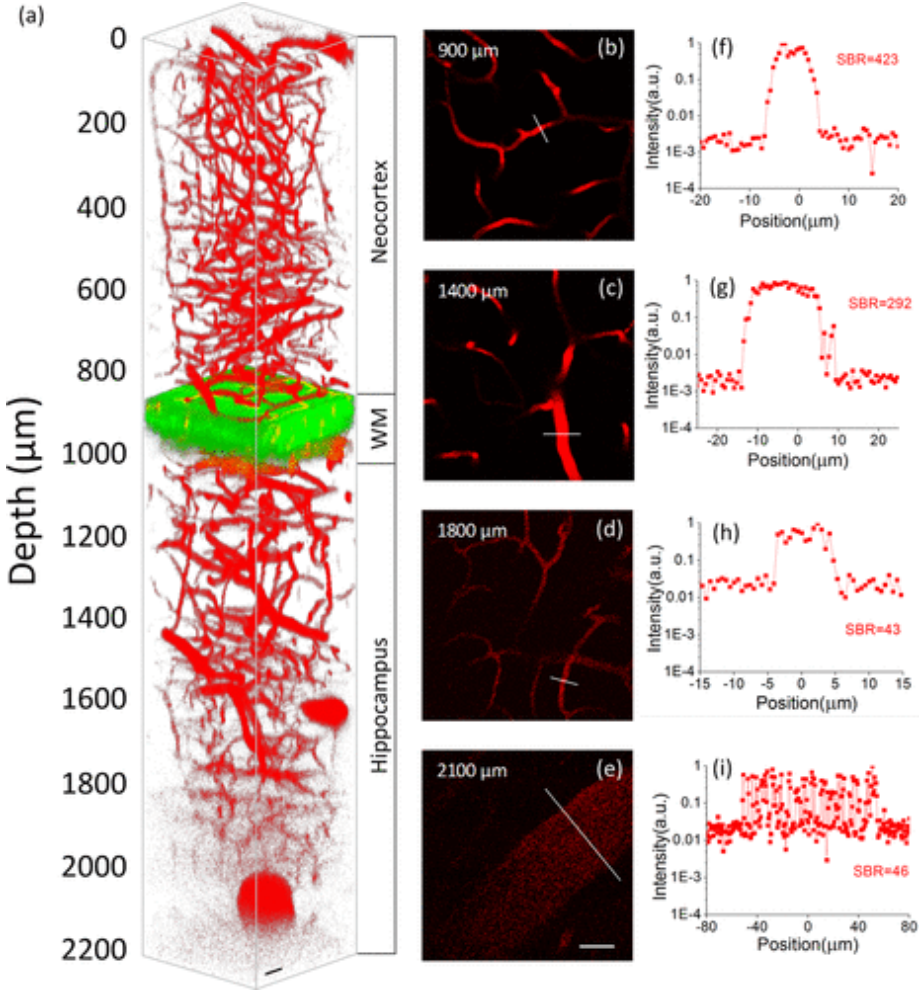
**2PE at 1280 nm[1]**




1280-nm EAL = 285 μm
SBR=1 @ z/EAL ~ 5

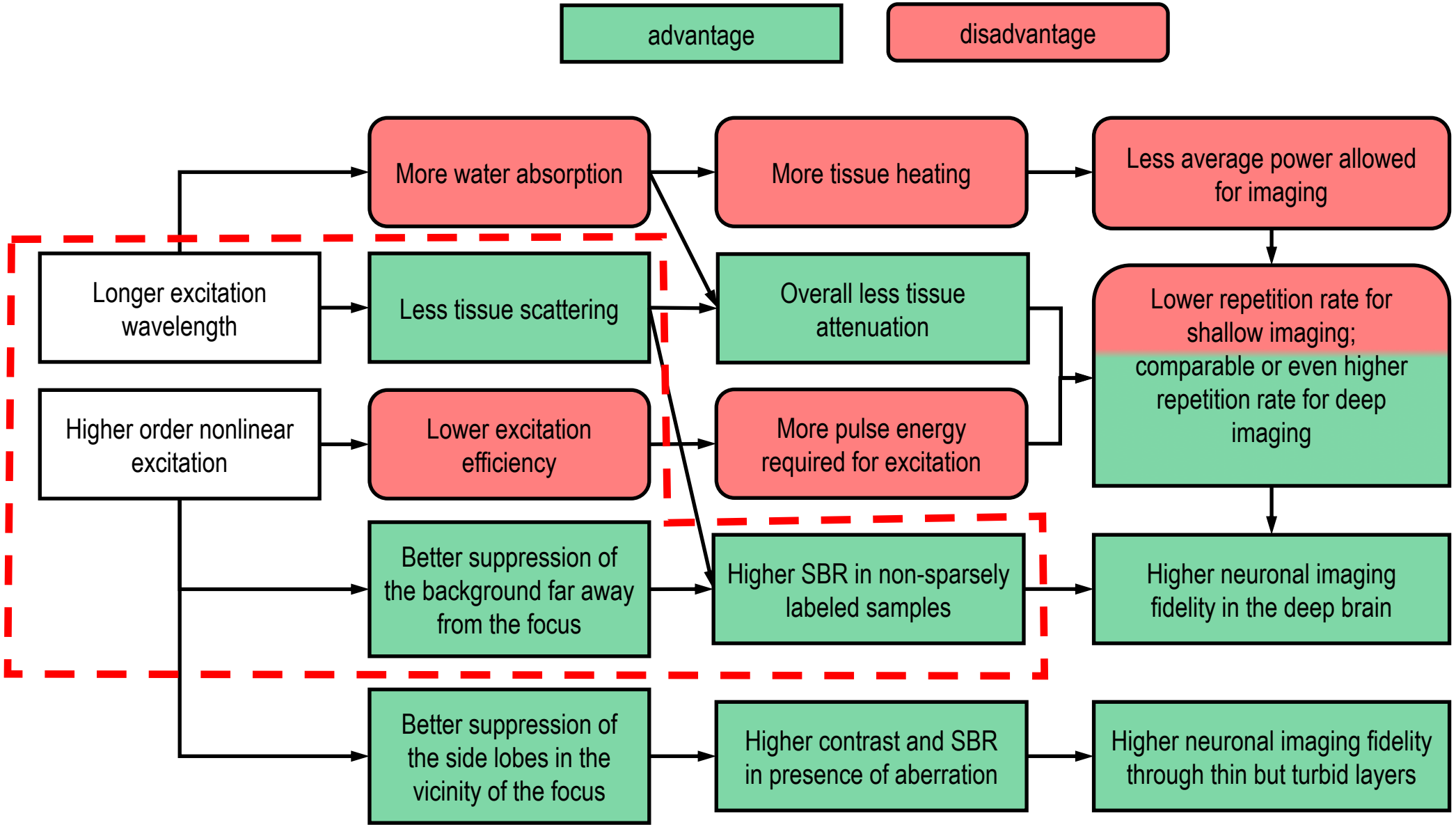
**3PE at 1700 nm[2]**




1700-nm EAL ~ 400 μm in cortex, ~1/2 in the white matter

SBR =46 @ z/EAL > 5


1. "*In vivo* two-photon microscopy to 1.6-mm depth in mouse cortex." *J. Biomed. Opt.* (Kobat..., C. Xu, 2011) https://doi.org/10.1117/1.3646209
2. "*In Vivo* Deep-Brain Structural and Hemodynamic Multiphoton Microscopy Enabled by Quantum Dots" *Nano Lett.* (H. Liu, .. P. Qiu, and K. Wang 2019) https://doi.org/10.1021/acs.nanolett.9b01708


advantage
disadvantage
More water absorption
More tissue heating
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Longer excitation wavelength
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Overall less tissue attenuation
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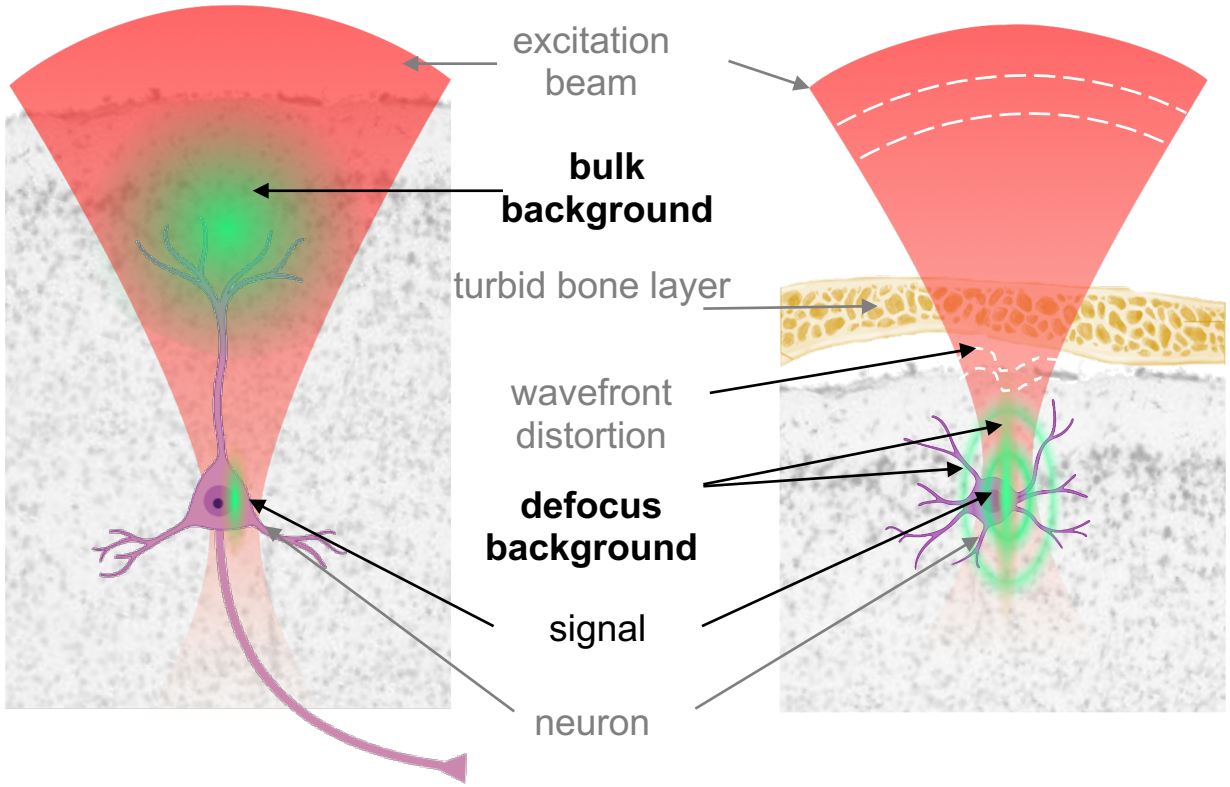
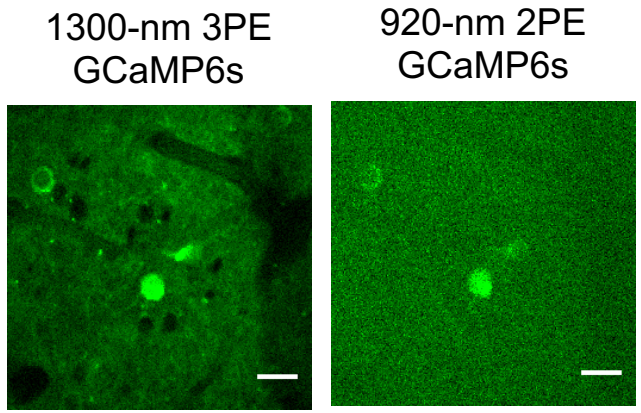
# Aberration Induced Background




- The degradation of focal quality by sample aberration creates *defocus background* that also reduces imaging quality.
- Defocus background happens in the vicinity of the focus, and is often not included in SBR calculation.
- This background is primarily determined by the sample aberration and can even be present at a shallow imaging depth.

"Three-photon neuronal imaging in deep mouse brain " *Optica* (T. Wang and C. Xu, 2020) https://doi.org/10.1364/OPTICA.395825

# Effects of Defocus Background on *in vivo* Imaging

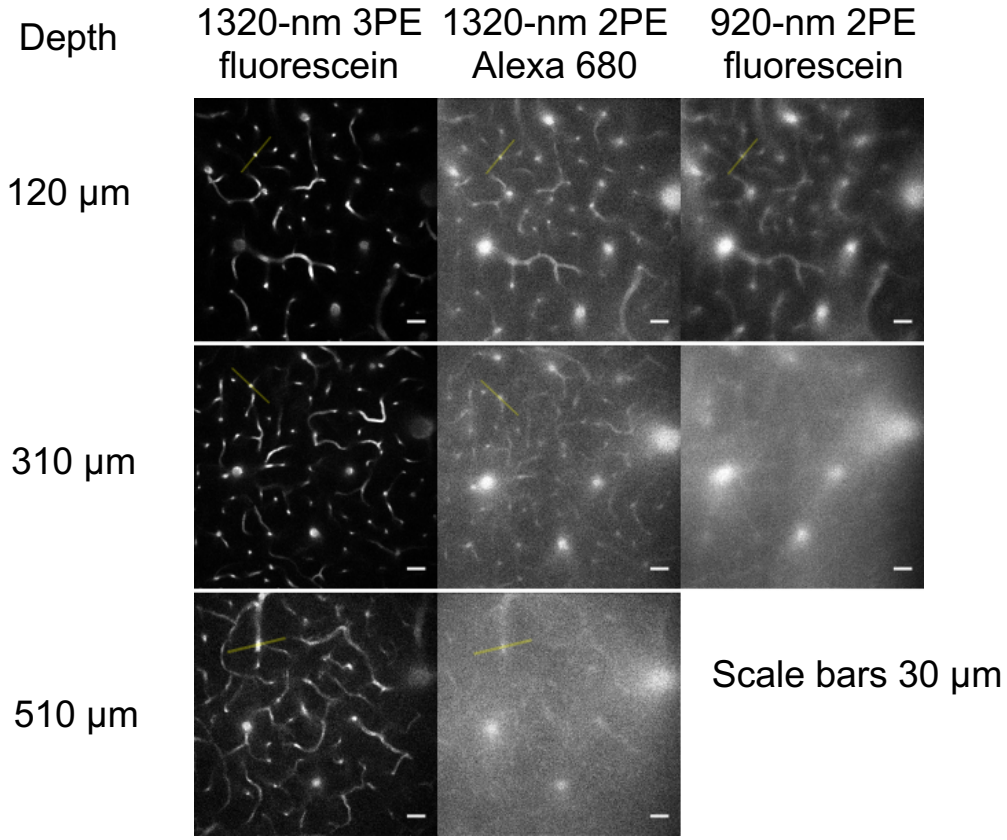


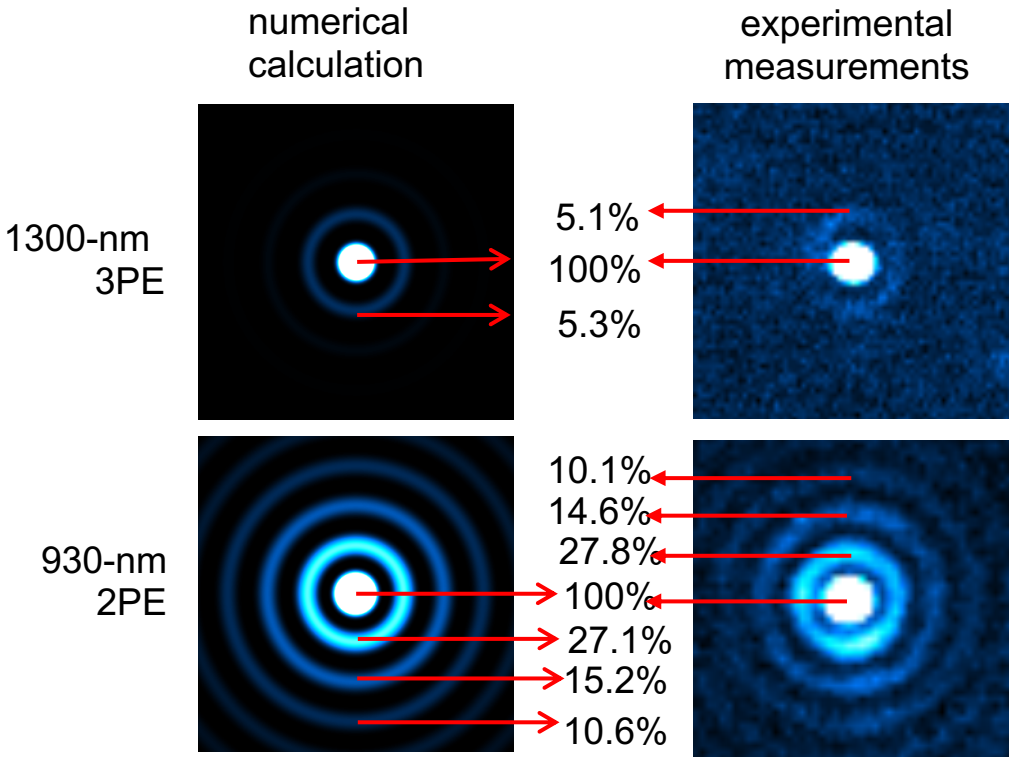

Bulk background: happens at only large depth, more homogeneous across the field-of-view.

Defocus background: can happen at a shallow depth, inhomogeneous across the field-of-view, with compromised feature resolution.

"Three-photon neuronal imaging in deep mouse brain " *Optica* (T. Wang and C. Xu, 2020) https://doi.org/10.1364/OPTICA.395825

# 3PE helps to suppress defocus background



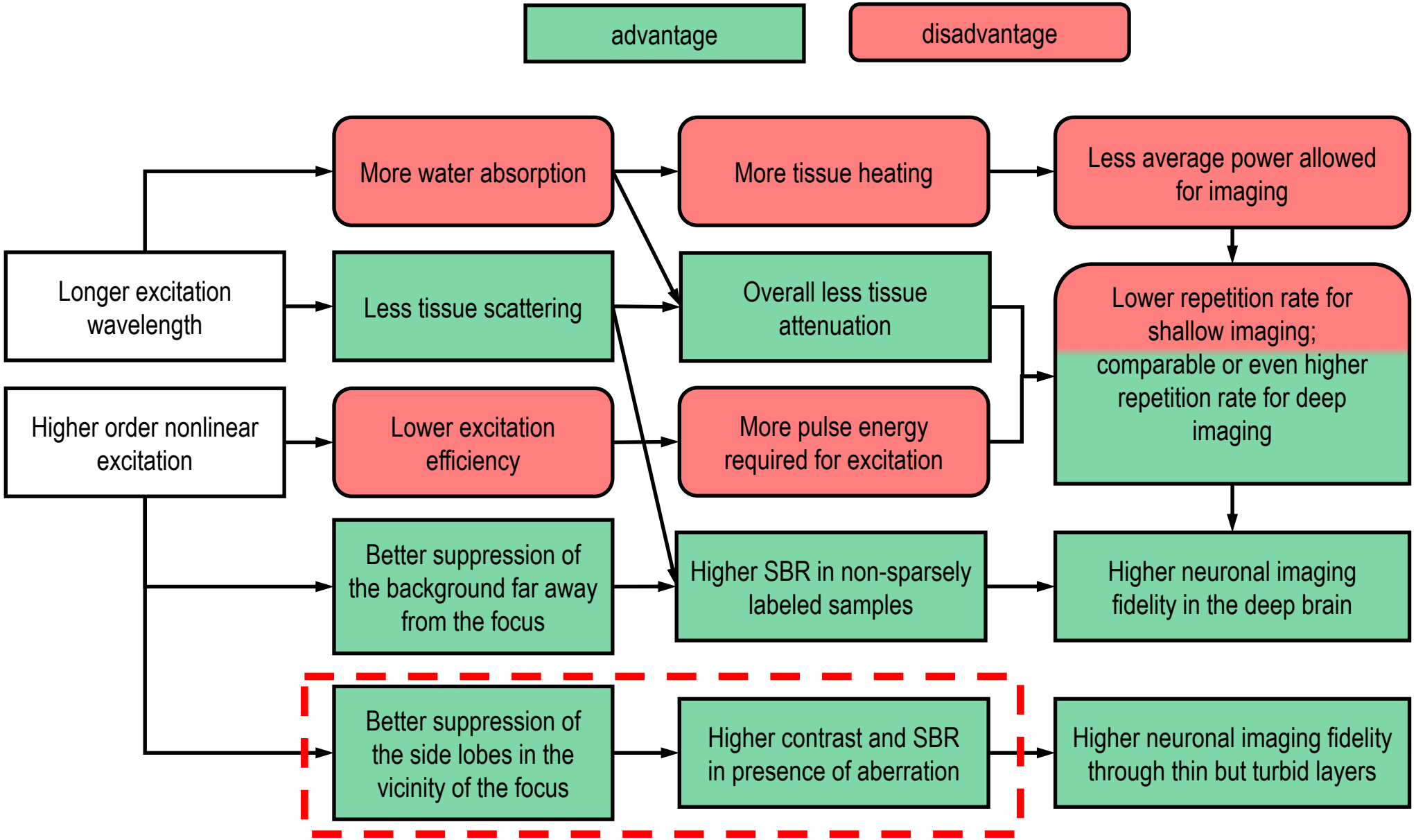




3P defocus background << 2P, with the same excitation beam and wavelength imaging different fluorophores with 2PE and 3PE respectively in the same sample.

"Three-photon imaging of mouse brain structure and function through the intact skull" *Nat. Methods* (T. Wang,, ..., C. Xu, 2018) https://doi.org/10.1038/s41592-018-0115-y

3P imaging with Bessel beam reveals that 3PE can suppress side lobes with $I^3$ dependence.

"Rapid volumetric imaging with Bessel-Beam three-photon microscopy" *Biomed. Opt. Express* (B. Chen, ...and A. Wang, 2018) https://doi.org/10.1364/BOE.9.001992


advantage
disadvantage
Longer excitation wavelength
Higher order nonlinear excitation
More water absorption
Less tissue scattering
Lower excitation efficiency
Better suppression of the background far away from the focus
Better suppression of the side lobes in the vicinity of the focus
More tissue heating
Overall less tissue attenuation
More pulse energy required for excitation
Higher SBR in non-sparsely labeled samples
Higher contrast and SBR in presence of aberration
Less average power allowed for imaging
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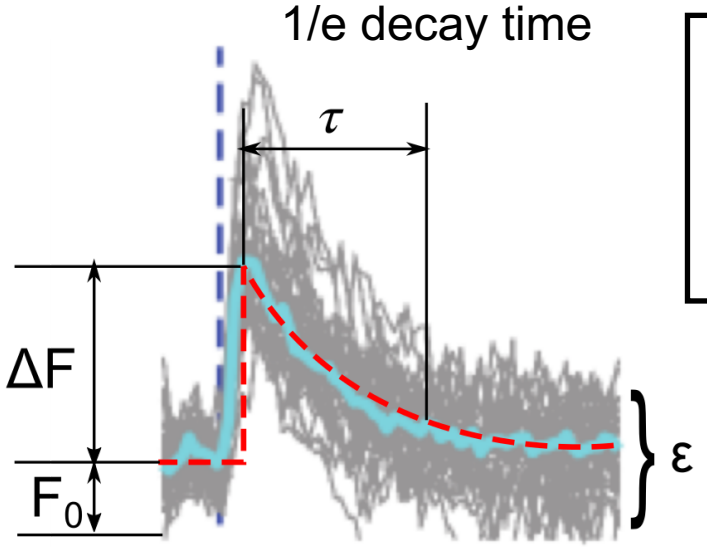
Higher neuronal imaging fidelity in the deep brain
Higher neuronal imaging fidelity through thin but turbid layers

# Calcium Spike Inference Model

Triggered by 1 action potential




Peak fluorescence change induced by 1 action potential

Baseline fluorescence intensity

$$\bar{F}(t) = F_0 + \Delta F e^{-t/\tau}\,\theta(t)$$

$$F(t) \sim Poission(\bar{F}(t))$$

$$F(t) \sim N(\bar{F}(t); \bar{F}(t)) \text{ for large } F_0$$

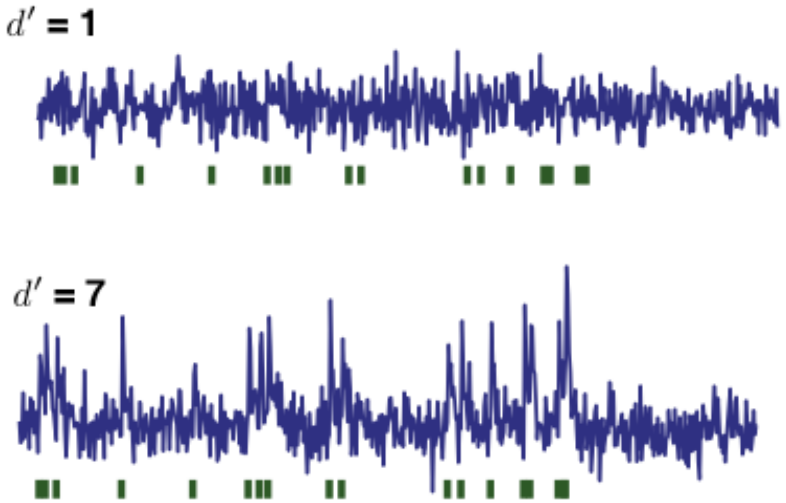
The parameters are determined by the calcium indicator characteristics.
For example, $\Delta F/F \sim 30\%$, $\tau \sim 2s$ for GCaMP6s*.

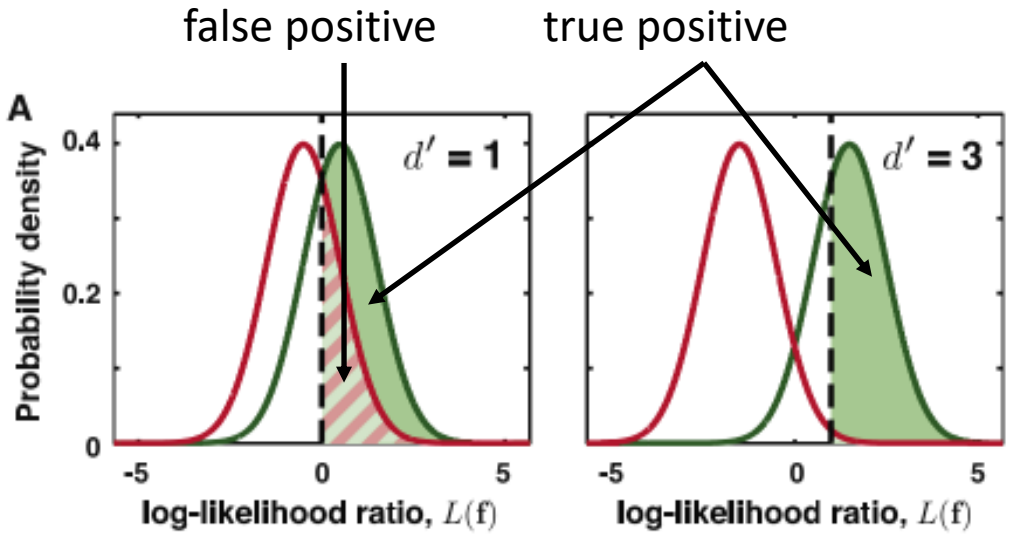
"Photon shot noise limits on optical detection of neuronal spikes and estimation of spike timing." (Wilt., Fitzgerald, & Schnitzer, 2013). *Biophysical Journal*. https://dx.doi.org/10.1016%2Fj.bpj.2012.07.058

* "Ultrasensitive fluorescent proteins for imaging neuronal activity." (Chen, T.-W., …, Looger, Svoboda, & Kim. 2013) *Nature* https://doi.org/10.1038/nature12354

# The Quality of Inference

- Spike detection is harder with noisier traces and the difficulty can be quantified by the $d'$ parameter:




$$d' \approx \frac{\Delta F}{F}\sqrt{\frac{F_0\tau}{2}} \text{ when sample rate} \gg 1/\tau$$

$d'$ = 3 gives 93% true positive and 7% false positive rate.
$d'$ = 5 gives 98.4% true positive and 1.6% false positive rate.

# Quantification of Neuronal Imaging Performance

- When $d'$ is viewed as a figure of merit, the performance of 3PM on neuronal imaging in deep brain becomes quantifiable.
- In particular, $d'$ is related to signal strength and SBR according to the following relation:

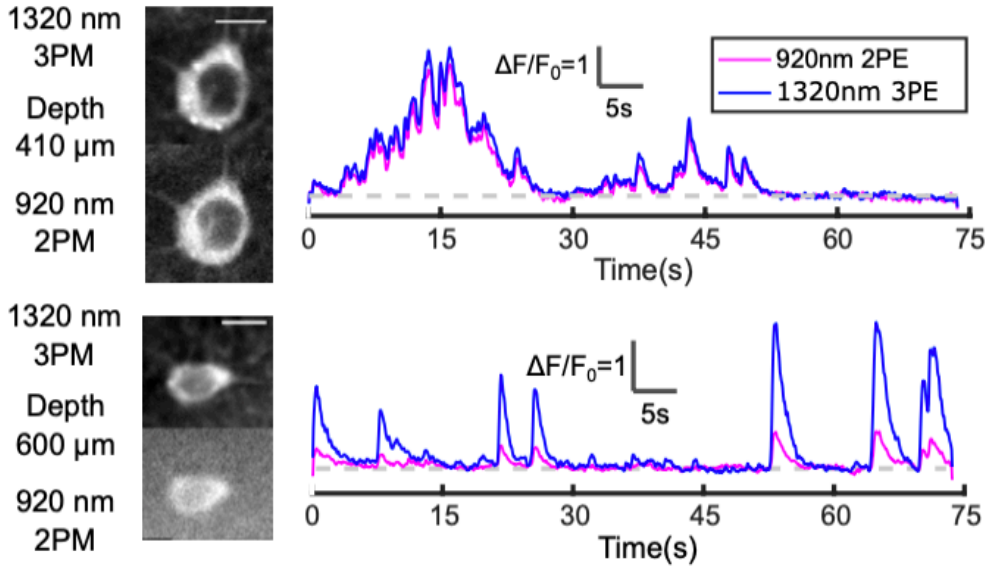
$$d' \approx \frac{1}{\sqrt{1 + 1/\mathrm{SBR}}} \frac{\Delta F}{F} \sqrt{\frac{F_0 \tau}{2}}$$

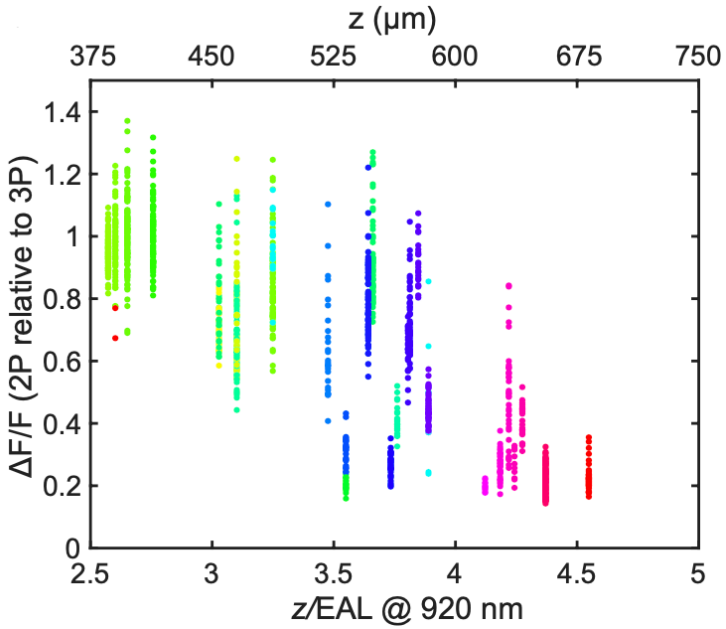
- Spike detection accuracy increases with $\sqrt{F_0 \tau}$, which scales with the S/N of the total number of photons ($F_0 \tau$).
- $d'$ decreases with background, since it reduces $\Delta F / F$ and increases the baseline noise.

"Quantitative analysis of 1300-nm three-photon calcium imaging in the mouse brain" *eLife* (T. Wang, C. Wu, …, C. Xu, 2020) https://doi.org/10.7554/eLife.53205

# 2P vs 3P Calcium Imaging Sensitivity $\Delta F/F$

- 2P and 3P $\Delta F/F$ of GCaMP6 are similar in the shallow cortex (up to 500 μm).
- The 2P $\Delta F/F$ drops down quickly for deeper imaging sites.

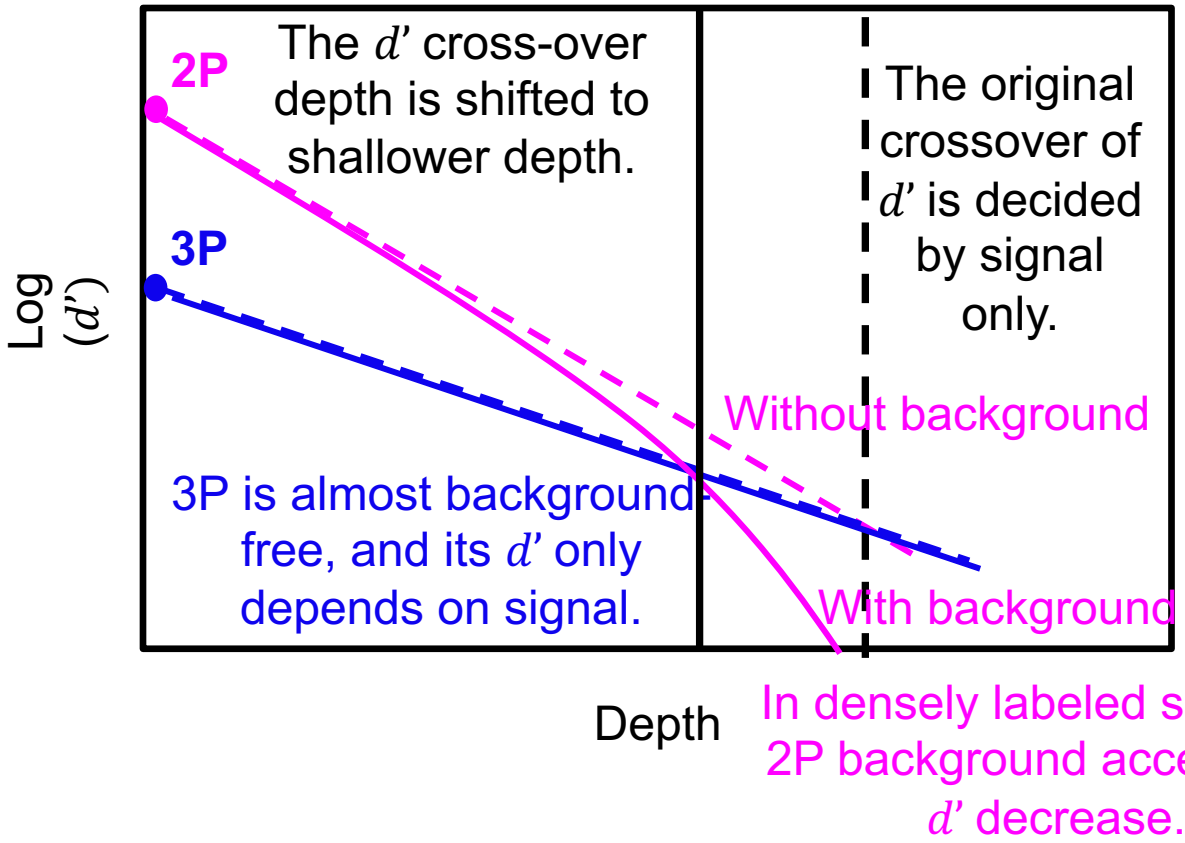


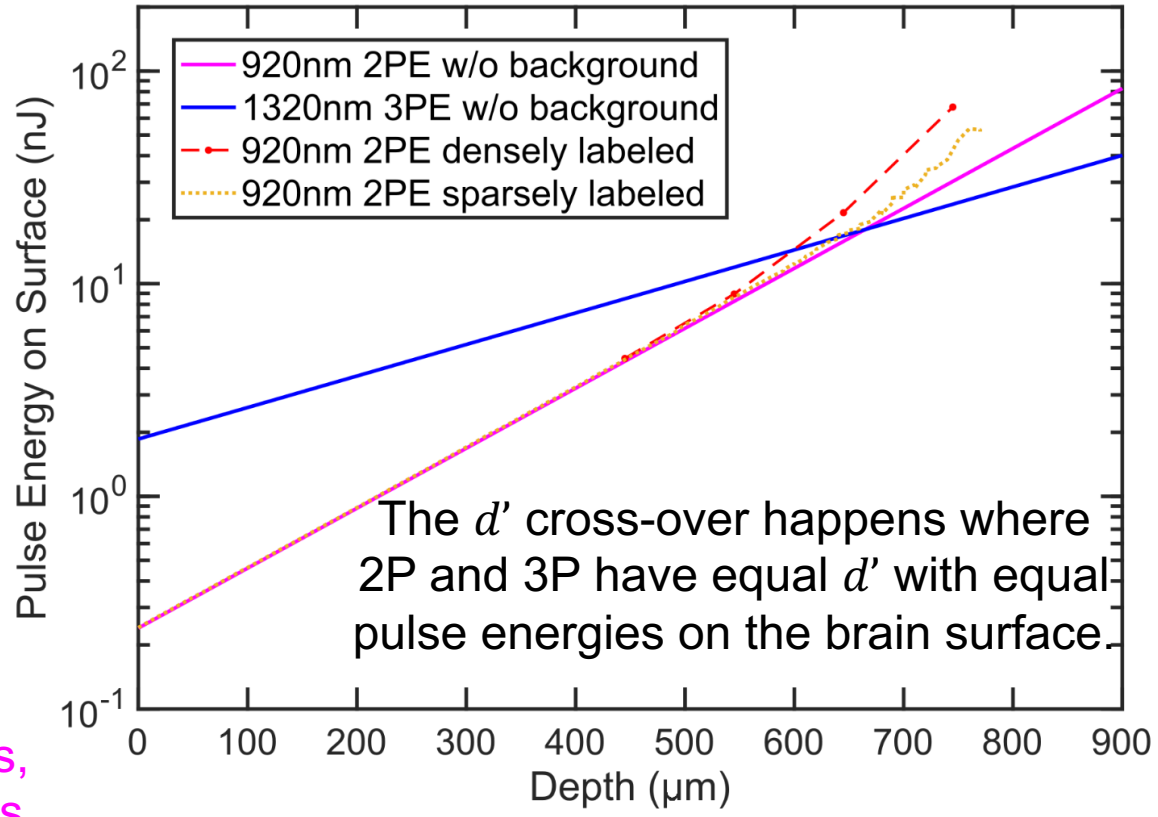




"Quantitative analysis of 1300-nm three-photon calcium imaging in the mouse brain" *eLife* (T. Wang, C. Wu, …, C. Xu, 2020) https://doi.org/10.7554/eLife.53205

# The Crossover Depth between 2P and 3P Neuronal Imaging

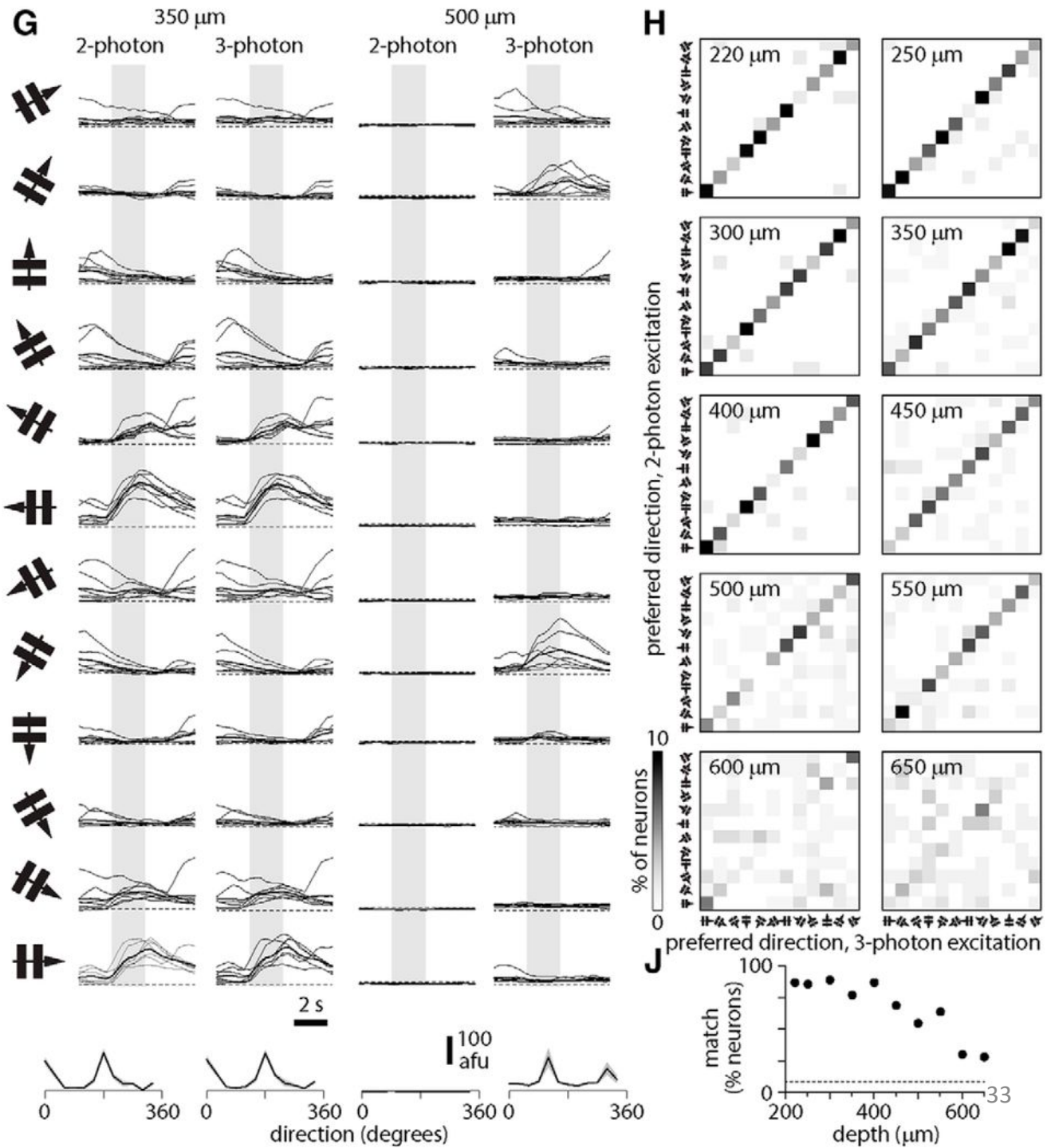
- $d'$ allows a comprehensive comparison between 2P and 3P neuronal imaging performance, taking both signal strength and background into account.



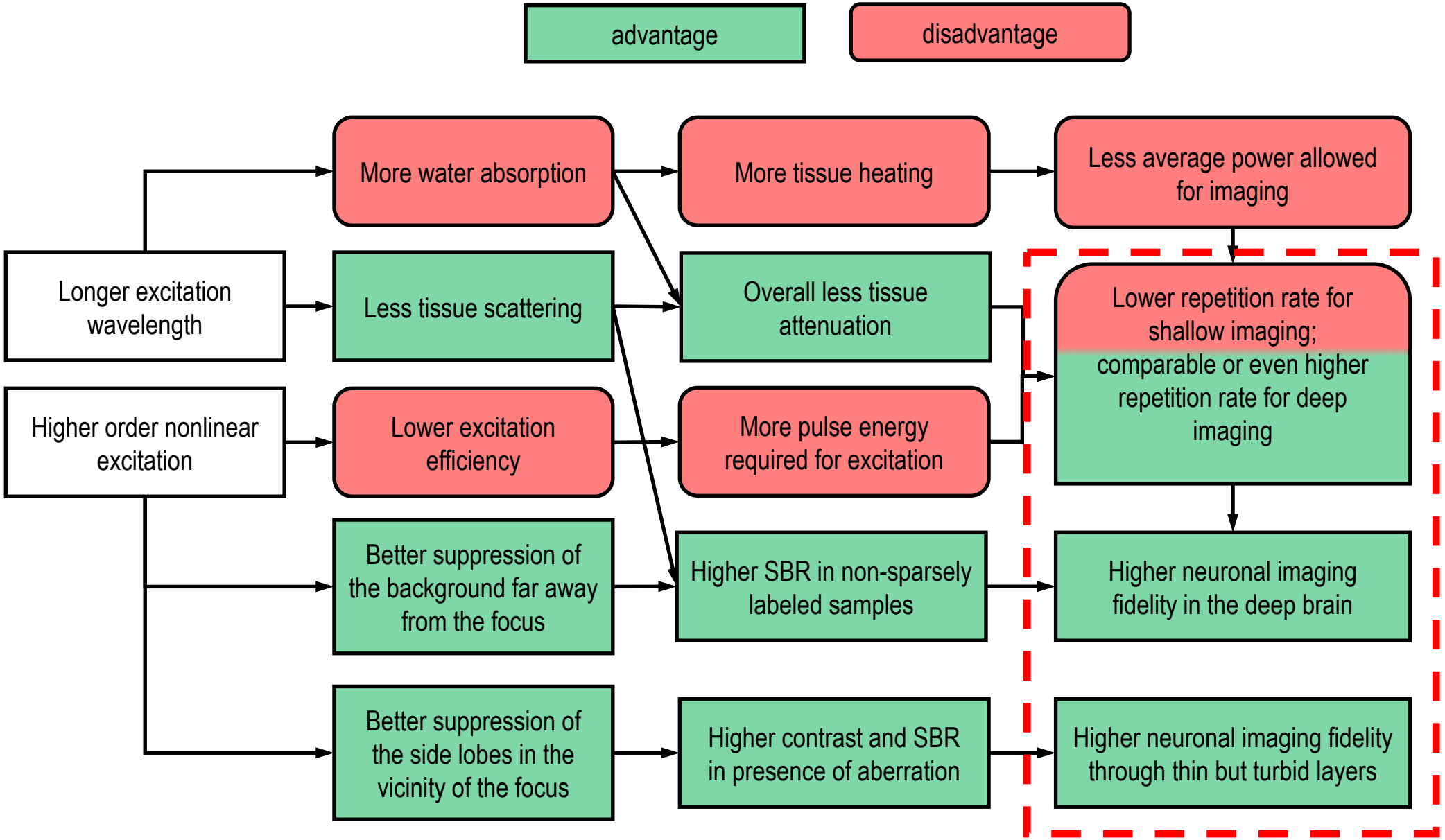



# Effects of Background on Neurophysiology Measurements

- In shallow regions of the brain, 2PM and 3PM yield the same results on visual orientation preference.
- For deep brain imaging, the high background of 2PM reduces the detectability of visual orientation tuning of neurons.
- This effect is probably more significant on dimmer neurons.




"Superficial Bound of the Depth Limit of Two-Photon Imaging in Mouse Brain" *eNeuro* (Takasaki..., Waters, 2020) https://doi.org/10.1523/ENEURO.0255-19.2019


advantage
disadvantage
Longer excitation wavelength
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More tissue heating
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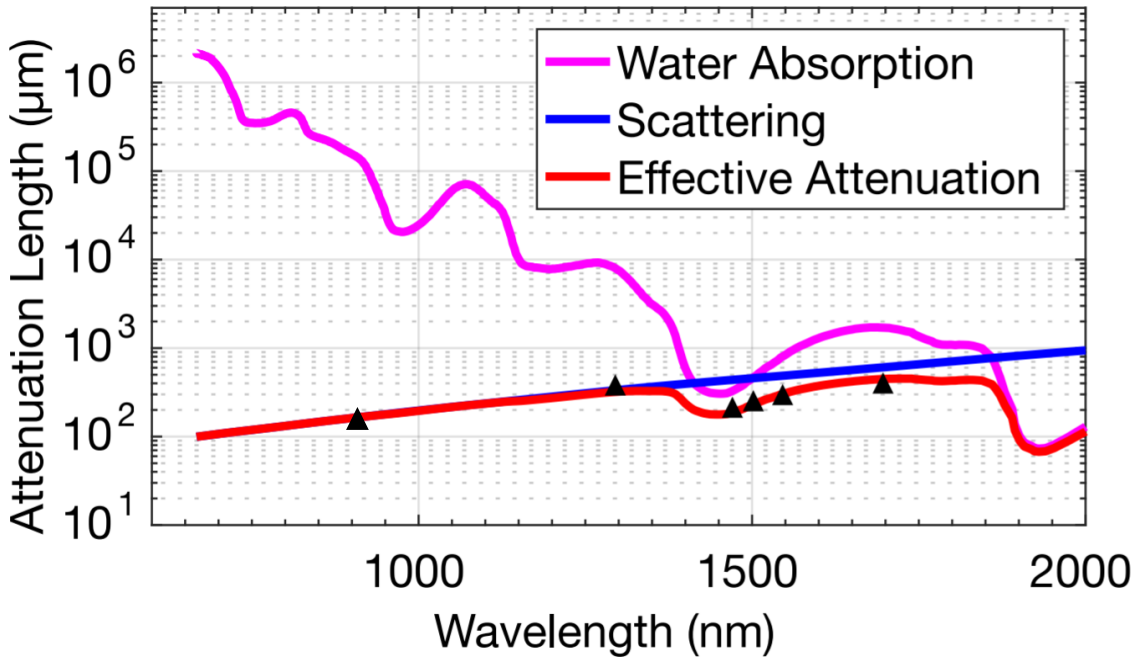
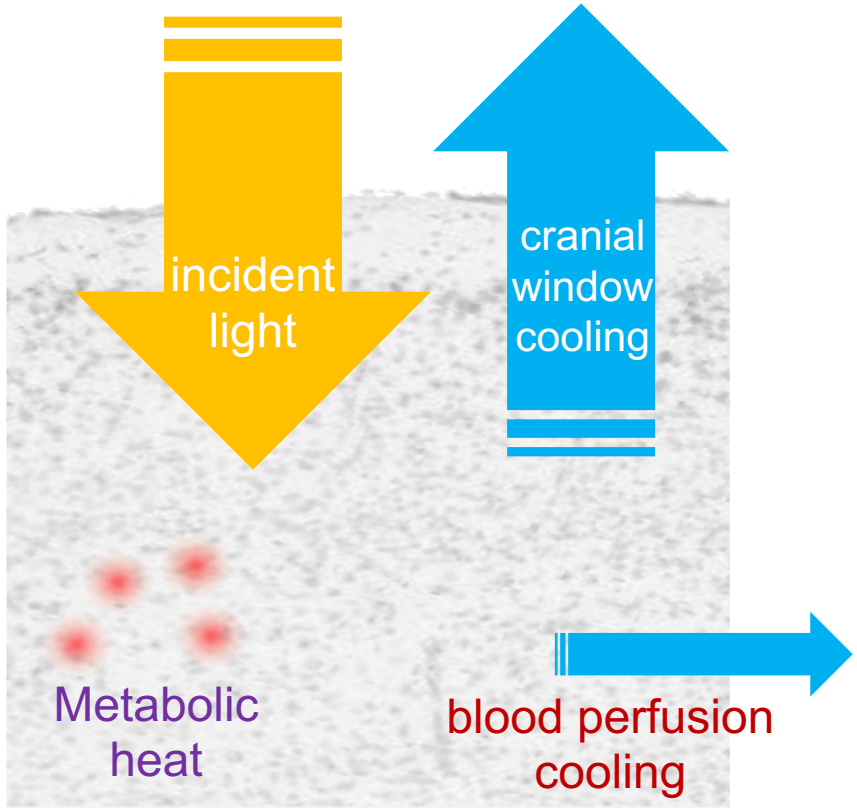
# Brain Heating by Average Power



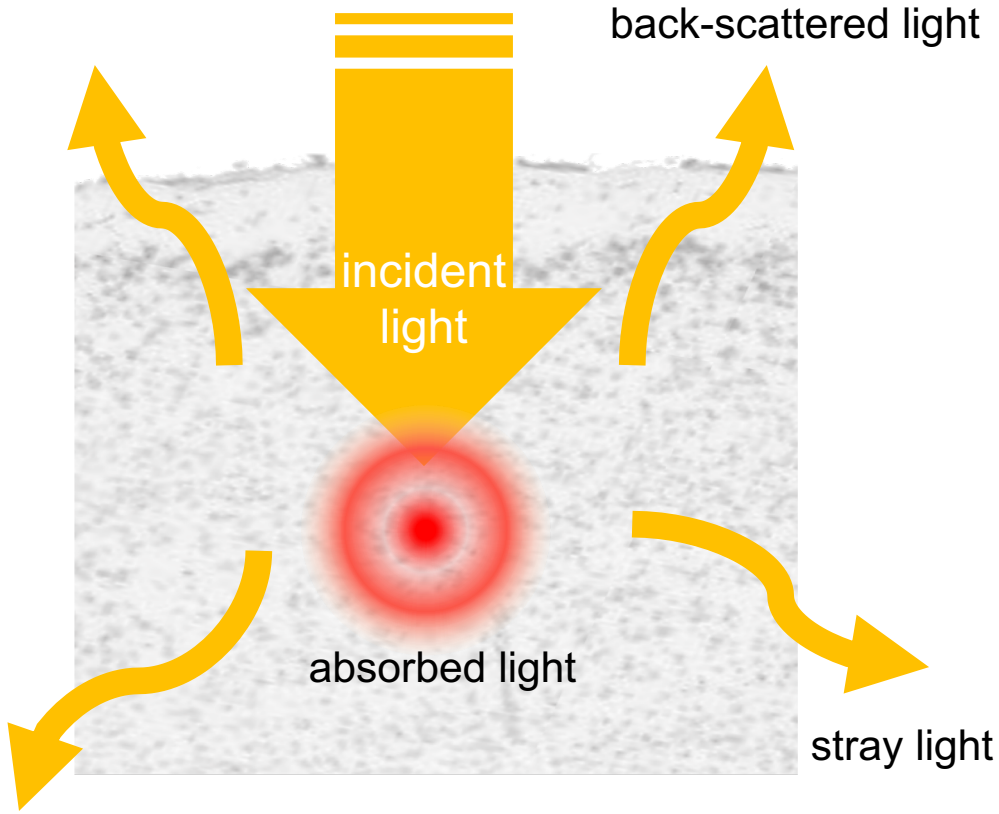

- Brain heating above the physiological temperature can cause change in neuron firing rate, denaturization of proteins, and even change of behavior.
- 3PE wavelengths experience higher water absorption than 2PE.
- The maximum allowable average power used for 3P imaging is lower than 2P.

# Brain Heating Model



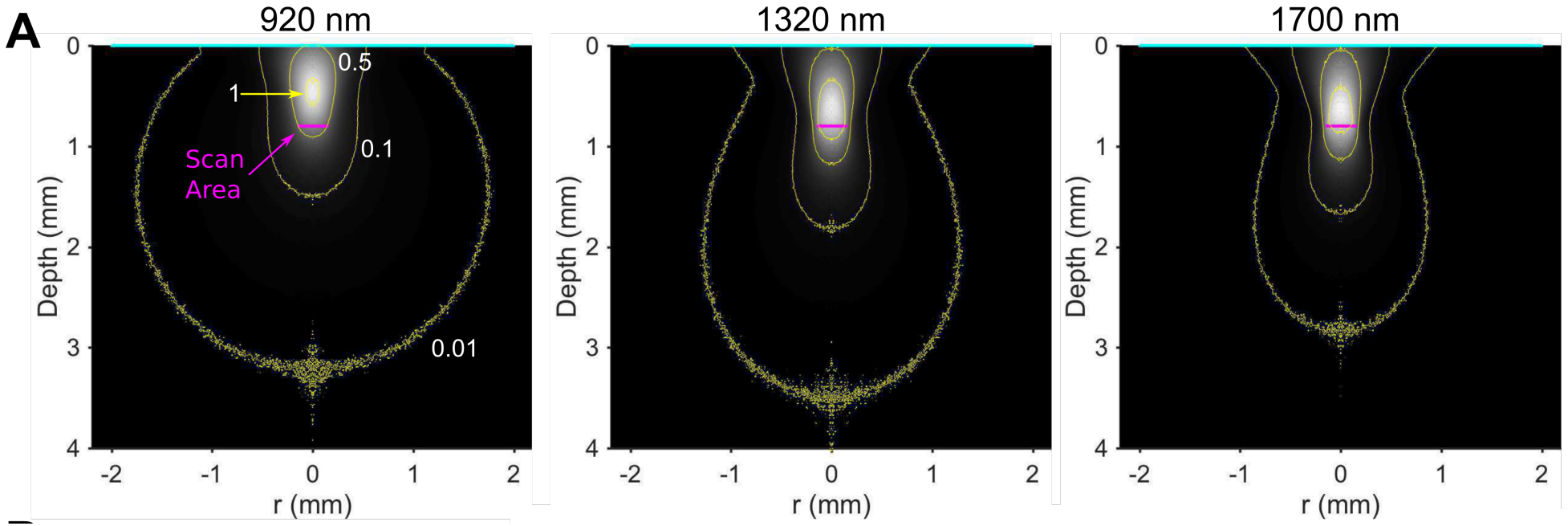

- The primary cooling source is the heat conduction through the cranial window, since the glass surface is maintained closer to room temperature.
- Without any external heating source, the brain surface temperature can be as low as ~32 °C
- Metabolic heat is not enough to maintain the brain temperature, and is approximately equal to the blood cooling rate.
- The brain heating starts when the light absorption rate > cranial window cooling.

# Tissue Light Transportation Model




- Only light absorbed around the hot spot contributes to brain heating.
- Other photons may be back-scattered out of the cranial window, or diffuse far away from the excitation volume.
- The percentage of light contributing to brain heating depends on the scattering and absorption coefficients.
- A higher percentage is absorbed for 3PE wavelengths than 2PE due to a higher ratio in absorption over scattering coefficients.

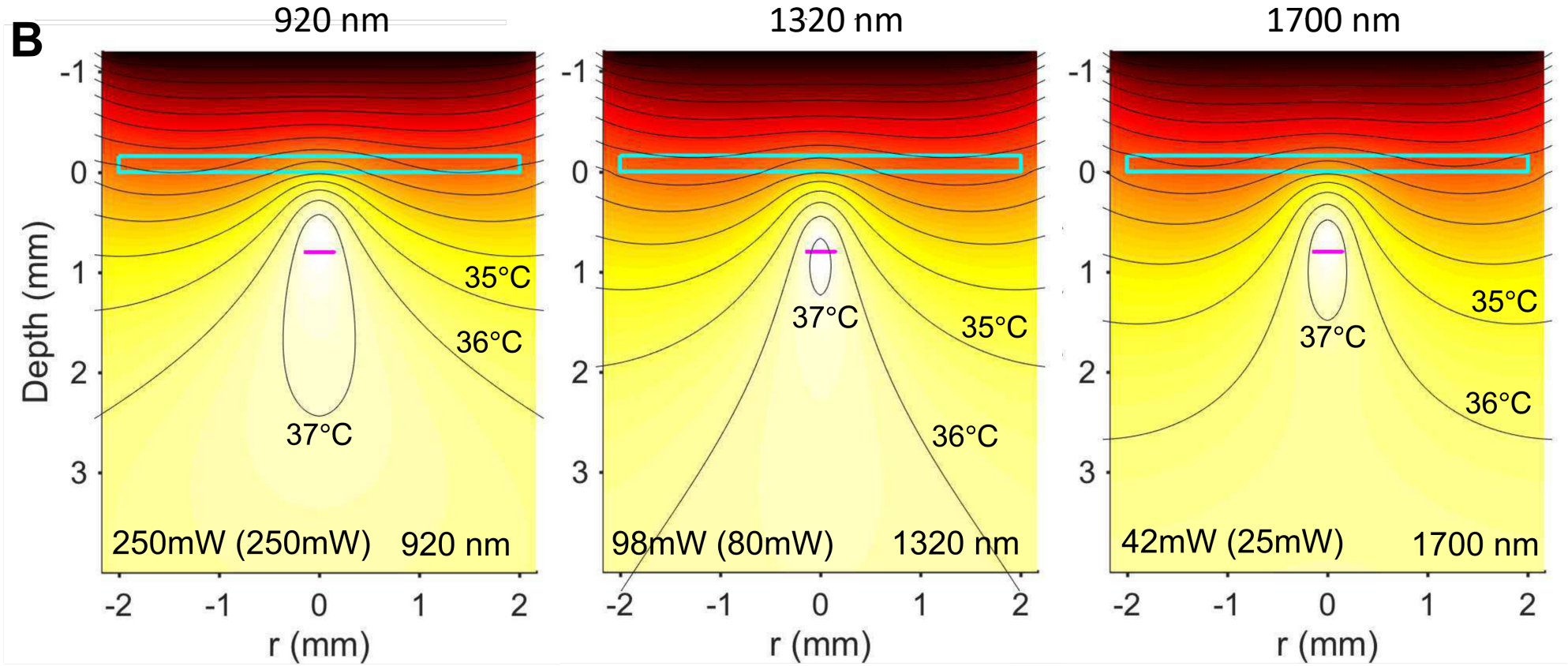
# Monte Carlo Simulation on Light Intensity Distribution




Simulated with tissue scattering and absorption coefficient at each wavelength.
The scanned area has a diameter of 0.3 mm at the depth of 0.8 mm.
A steady state is assumed for the simulation.

"CALCIUM IMAGING WITH THREE-PHOTON MICROSCOPY IN THE MOUSE BRAIN" *PhD thesis* (T. Wang, 2018)
https://doi.org/10.7298/X4Q81BBJ

# Simulation on Temperature Distribution in the Brain Tissue

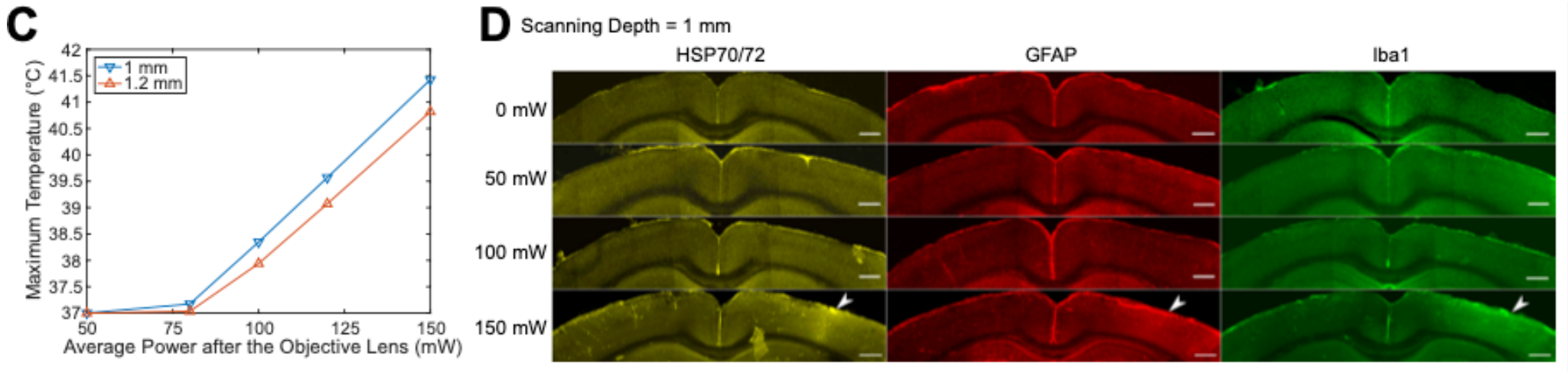



The temperature profile was calculated based on the light intensity distribution in the last slide

Two average powers are given: one after the objectives lens, the other one in the bracket is on the brain surface, this is because the attenuation of power by immersion water is not negligible for long wavelength.

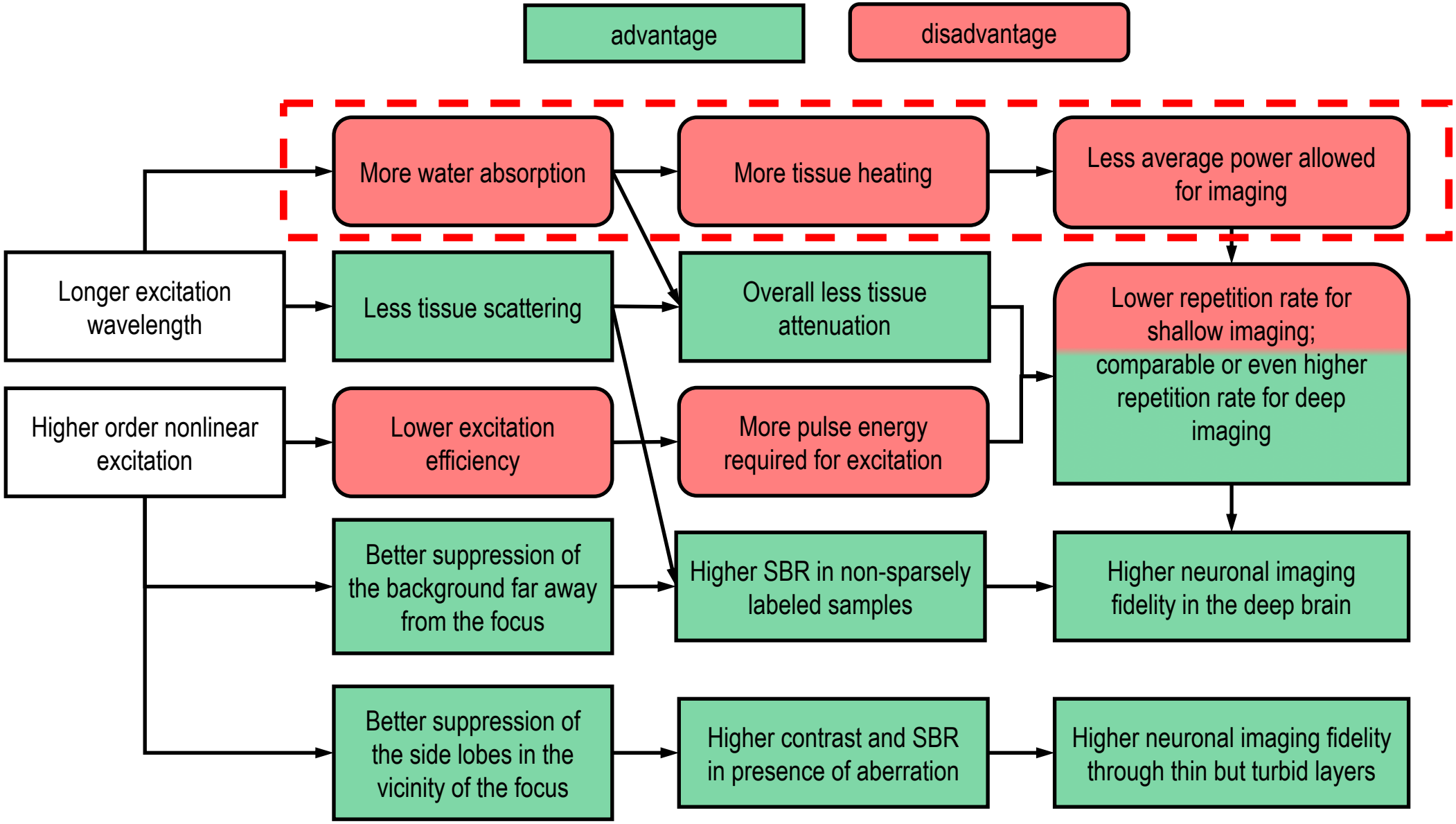
# Heating Damage and Peak Temperature for 1300-nm 3PM

- Immunohistochemistry assay was used to detect immune response to damage at given imaging powers and simulated peak temperature.
- The assay is identical to one employed to detect brain damage induced by 2PM.
- Brain damage can be detected at 150 mW average power for 1300-nm 3PM.
- Generally, 250 mW is considered safe for 2PM with 780-1060 nm excitation, since no damage was detected. The same can be said for 100 mW with 1300-nm 3PM.




"Brain heating induced by near-infrared lasers during multiphoton microscopy" *J. Neurophysiol* (Podgorski, …, Ranganathan, 2016) https://doi.org/10.1152/jn.00275.2016

"Quantitative analysis of 1300-nm three-photon calcium imaging in the mouse brain" *eLife* (T. Wang, C. Wu, …, C. Xu, 2020) https://doi.org/10.7554/eLife.53205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# Nonlinear Effects of 3PE

Undesired nonlinear effects induced by the high intensity of 3PE include:

- Nonlinear damage to the tissue
- Saturation of fluorophore
- Bleach of fluorophore

According to experimental results, the first two effects happen at approximate the same intensity level.

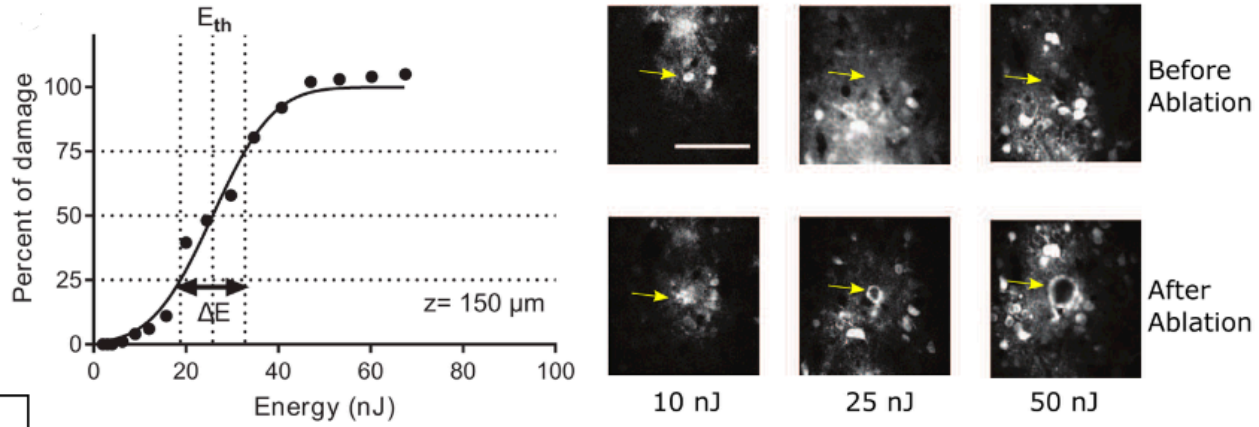
# Fluorophore Saturation

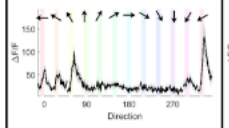
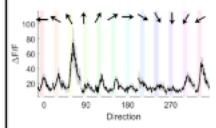
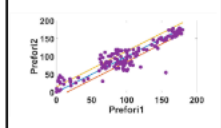
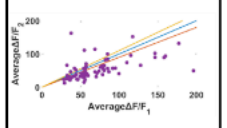
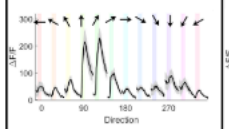
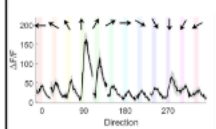
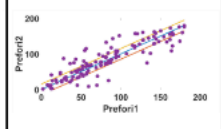
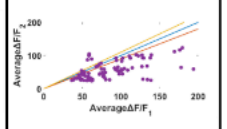
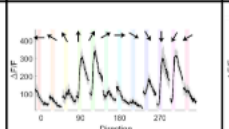
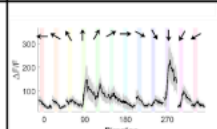
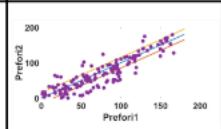
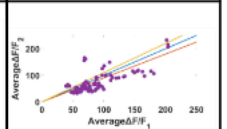
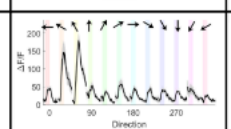
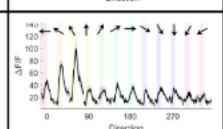
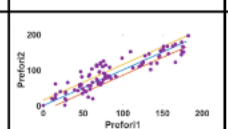
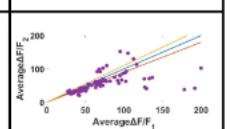
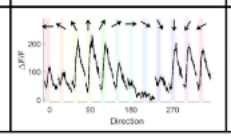
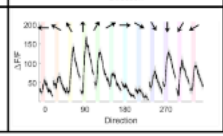
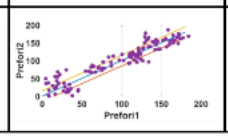
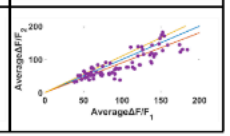
- Saturation happens when an intense excitation pulse excites most of the fluorophores in the focal volume.
- Saturation causes 3PE signal to fall below $I^3$ dependence, and has been experimentally observed to result in a lower apparent $\Delta F/F$ in calcium imaging.
- Saturation level can be quantified by the probability of exciting each fluorescent molecule per excitation pulse.
- Under common imaging conditions (NA~0.7, 60 fs)*, ~10% of GCaMP6 molecules are excited by a 2-nJ excitation pulse.
- Noticeably, 2 nJ generates about 0.1 photons per pulse in GCaMP6-labeled neurons (see ref. and slide 13).

"Quantitative analysis of 1300-nm three-photon calcium imaging in the mouse brain" *eLife* (T. Wang, C. Wu, …, C. Xu, 2020) https://doi.org/10.7554/eLife.53205

# Nonlinear Tissue Damage

- Nonlinear tissue damage happens at the focus and depends on excitation peak intensity in a highly nonlinear fashion.
- There is none-zero chance of visually identifiable tissue damage at as low as ~2 nJ (NA=1, 40fs) for 1300-nm 3PM.




| Layer/Energy | 0.5-2 nJ | 2-5 nJ | PrefOri | Average intensity |
|---|---|---|---|---|
| L 2/3 |  |  |  |  |
| L 4 |  |  |  |  |
| L 5 |  |  |  |  |
| L 6 |  |  |  |  |
| WM |  |  |  |  |

- Increasing the pulse energy to 2-5 nJ from 0.5-2 nJ changes 30% of neurons' orientation preference across all layers.
- The response strength is also reduced (probably due to saturation).

"Functional imaging of visual cortical layers and subplate in awake mice with optimized three-photon microscopy
" *Nat. Comm.* (Yildirim, .. P. T. C. So, and M. Sur 2019)
https://doi.org/10.1038/s41467-018-08179-6

- Introduction
- 2PM vs 3PM for Deep Neuronal Imaging
- The Framework for Optimizing 3PM for Large-volume Imaging

# Neuronal Imaging as An Optimization Problem

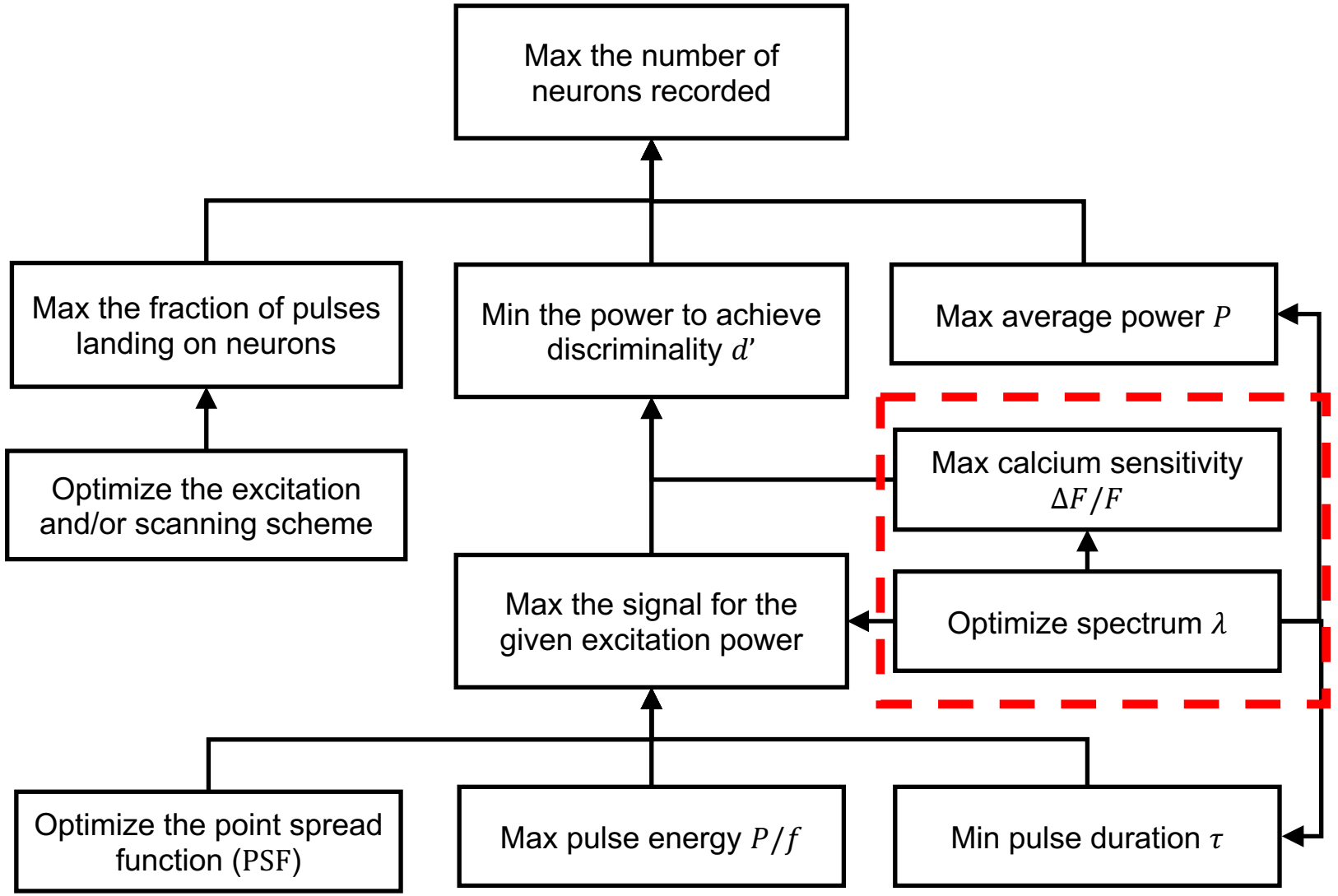
*Merit function*: maximize $d'$ times the number of neurons recorded (if we are only interested in spike detection)

*Constraints* (must be first measured and understood before optimization):

- Average power restricted by brain heating
- Peak intensity restricted by nonlinear effects

*Parameters*:

- Excitation pulse energy, pulse duration, repetition rate, and timing.
- Scanning field-of-view, scanning speed, and focal geometry.
- If the required parameter does not fall within the current technical capacity, it is a direction for future development to thrive on.

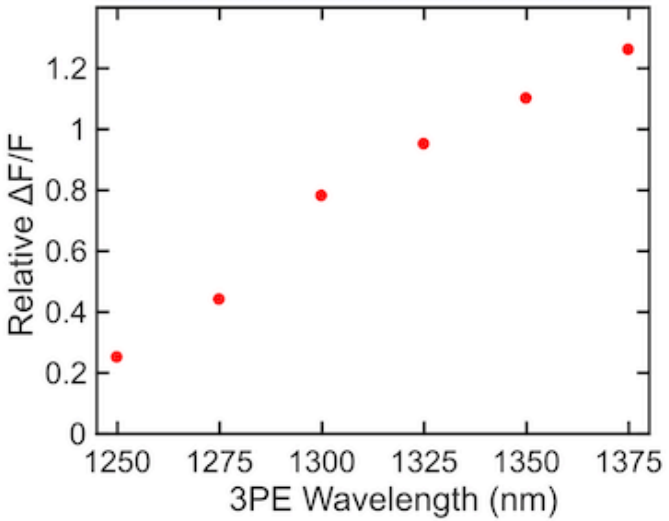

Max the number of neurons recorded
Max the fraction of pulses landing on neurons
Min the power to achieve discriminality $d'$
Max average power $P$
Optimize the excitation and/or scanning scheme
Max calcium sensitivity $\Delta F/F$
Max the signal for the given excitation power
Optimize spectrum $\lambda$
Optimize the point spread function (PSF)
Max pulse energy $P/f$
Min pulse duration $\tau$

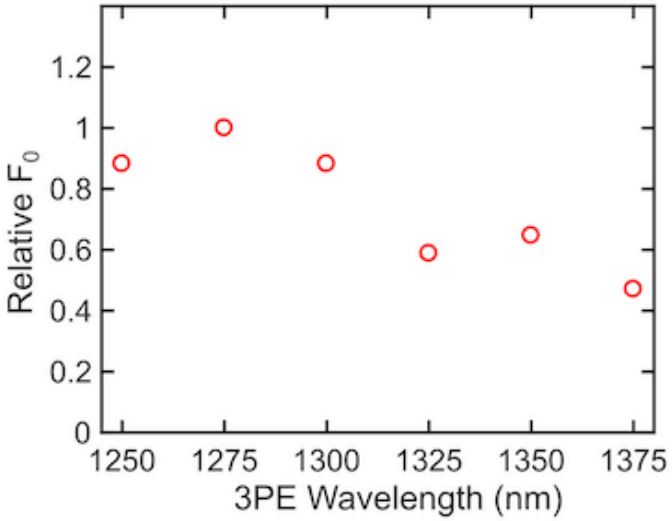
# Optimization of Excitation Wavelength

3PE wavelength affects neural imaging in several ways:

- calcium indicator sensitivity $\Delta F/F$
- calcium indicator brightness $F_0$
- tissue absorption of light
- spectrum width affects pulse duration

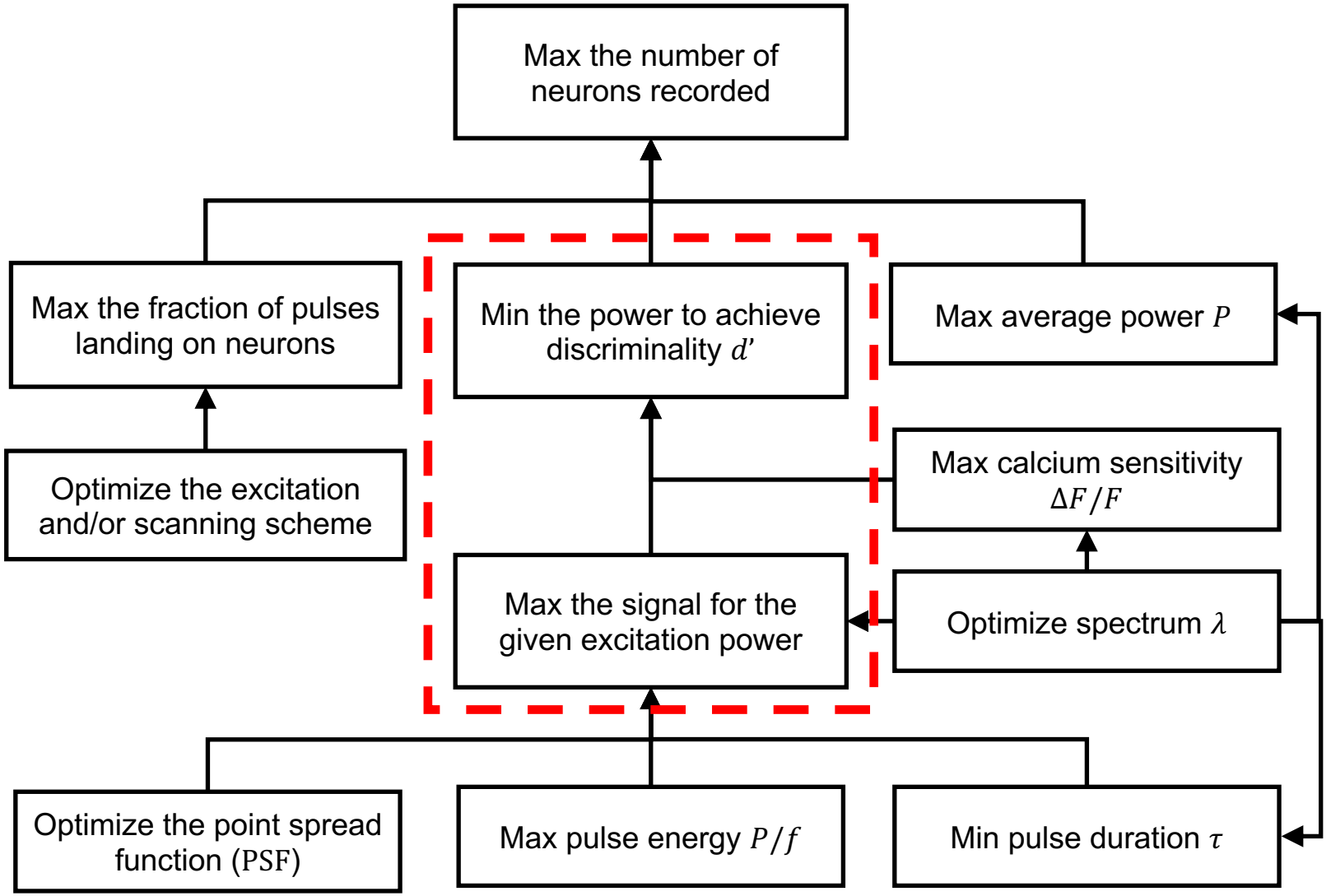
Overall, a spectrum centered at 1300-1320 nm with ~60 nm FWHM and well-managed dispersion is optimal for 3P imaging of GCaMP6.



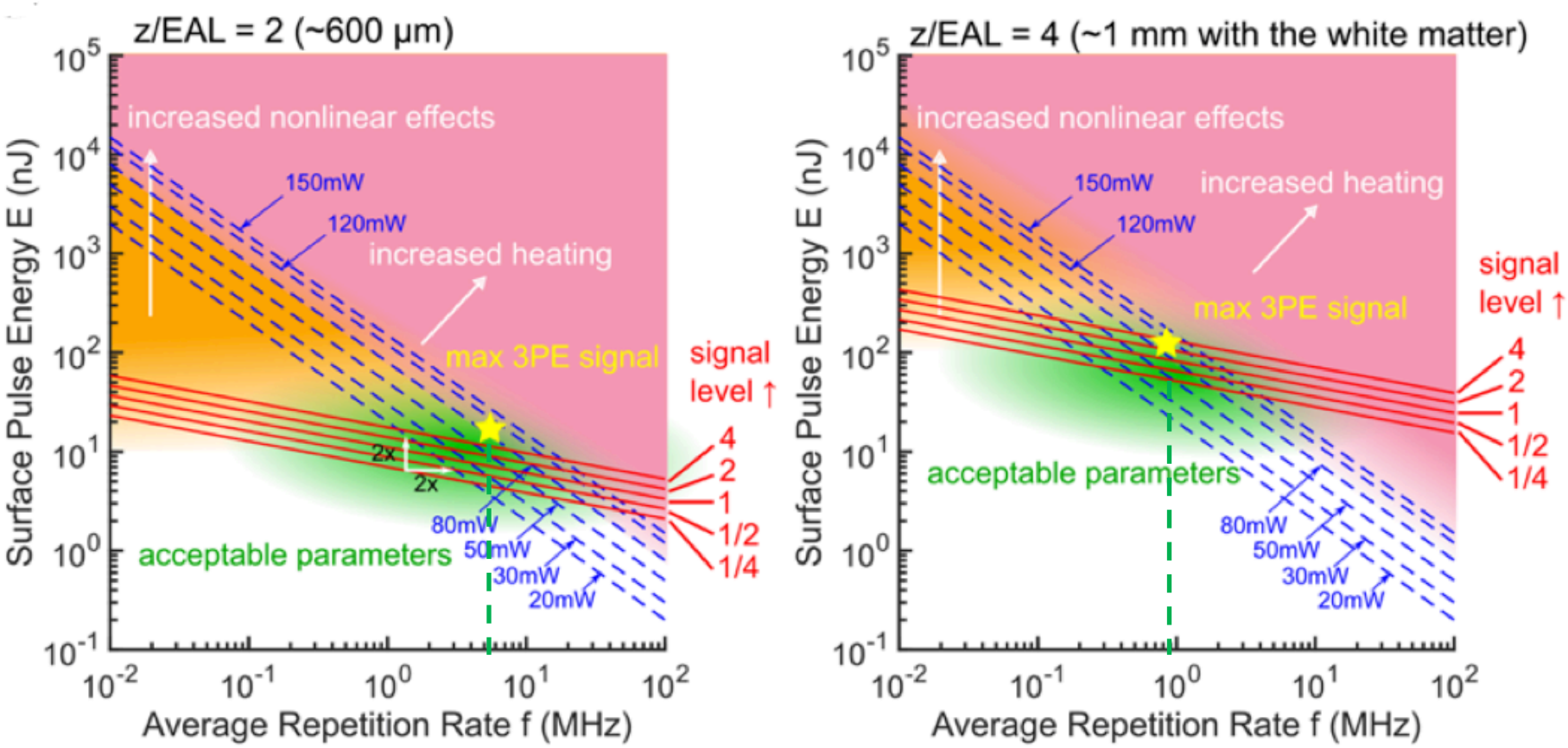

"GCaMP6 ΔF/F dependence on the excitation wavelength in 3-photon and 2-photon microscopy of mouse brain activity" *Nat. Comm.* (Ouzounov, .. C. Xu 2019) https://doi.org/10.1364/BOE.10.003343

https://www.janelia.org/lab/harris-lab/research/photophysics/two-photon-fluorescent-probes



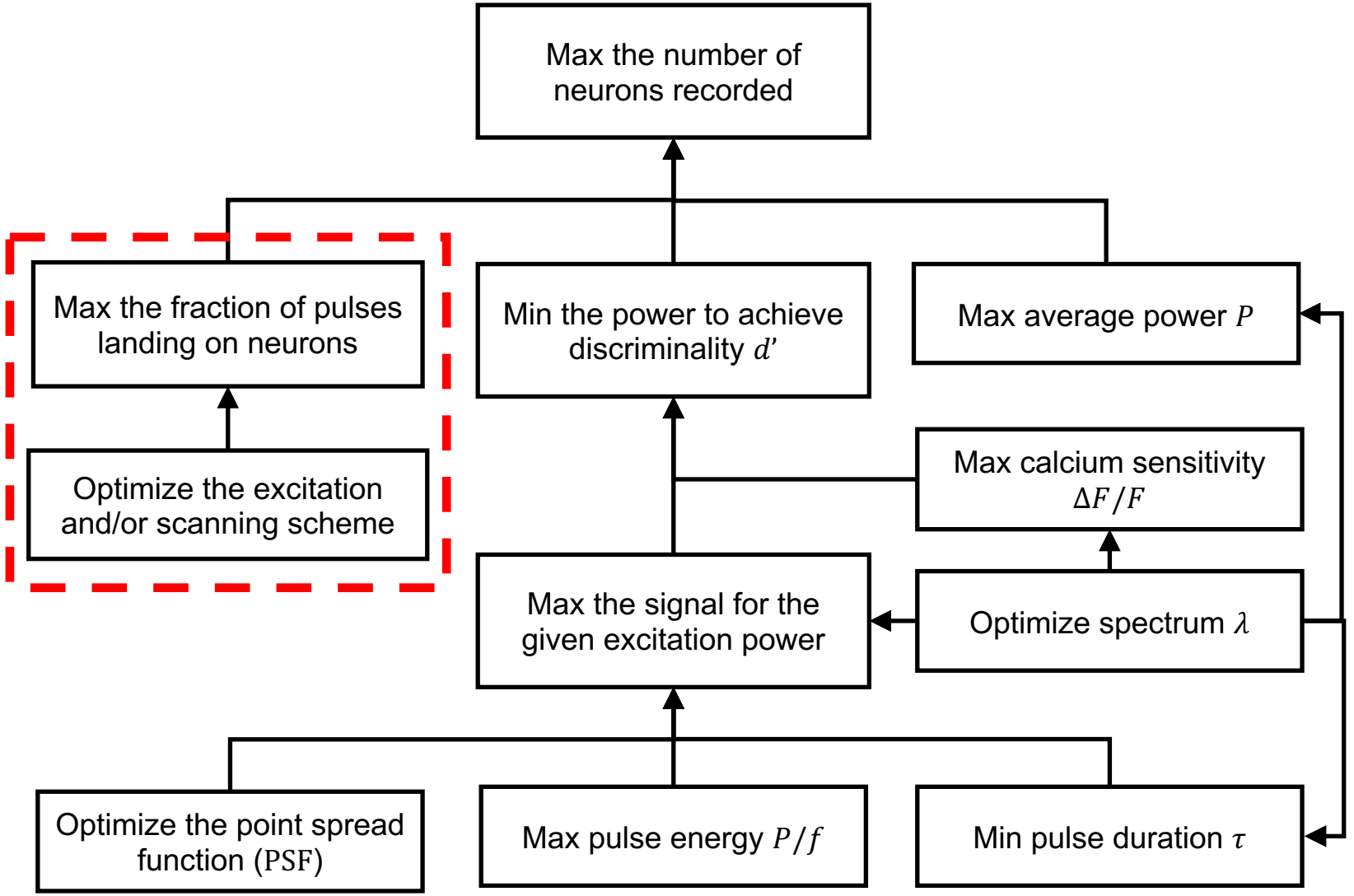

$$d' \approx \frac{1}{\sqrt{1+1/\mathrm{SBR}}} \frac{\Delta F}{F} \sqrt{\frac{F_0 \tau}{2}}$$

# Optimization of Repetition Rate to Imaging Depth




$$\text{3PE signal} \propto \left(\frac{P_{3P}}{f}\right)^3 f$$

$$\text{Pulse energy on sample surface} = \frac{P_{3P}}{f} = \text{pulse energy at the focus} \times \exp\left(\frac{z}{EAL}\right)$$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Max pulse energy $P/f$
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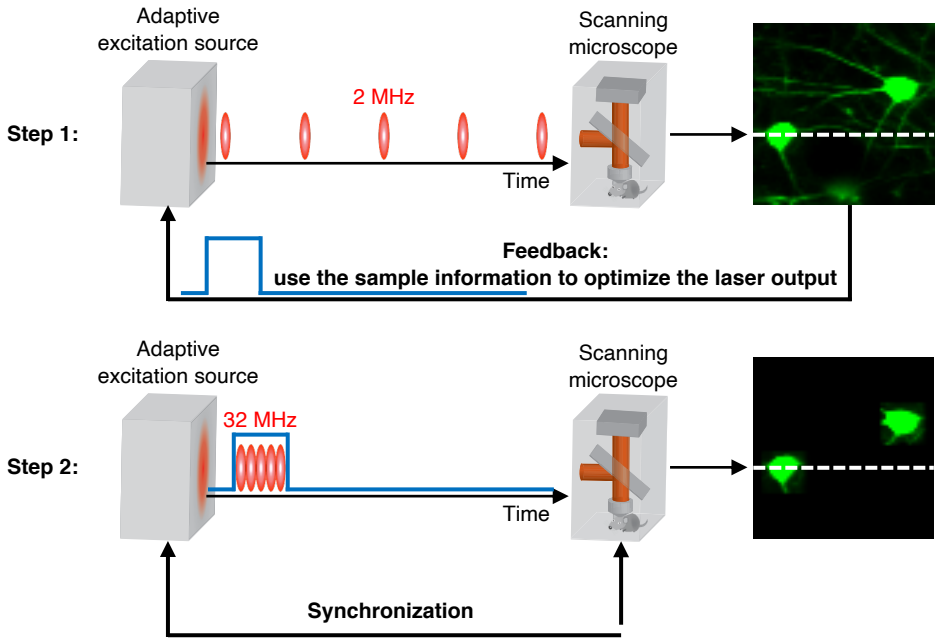
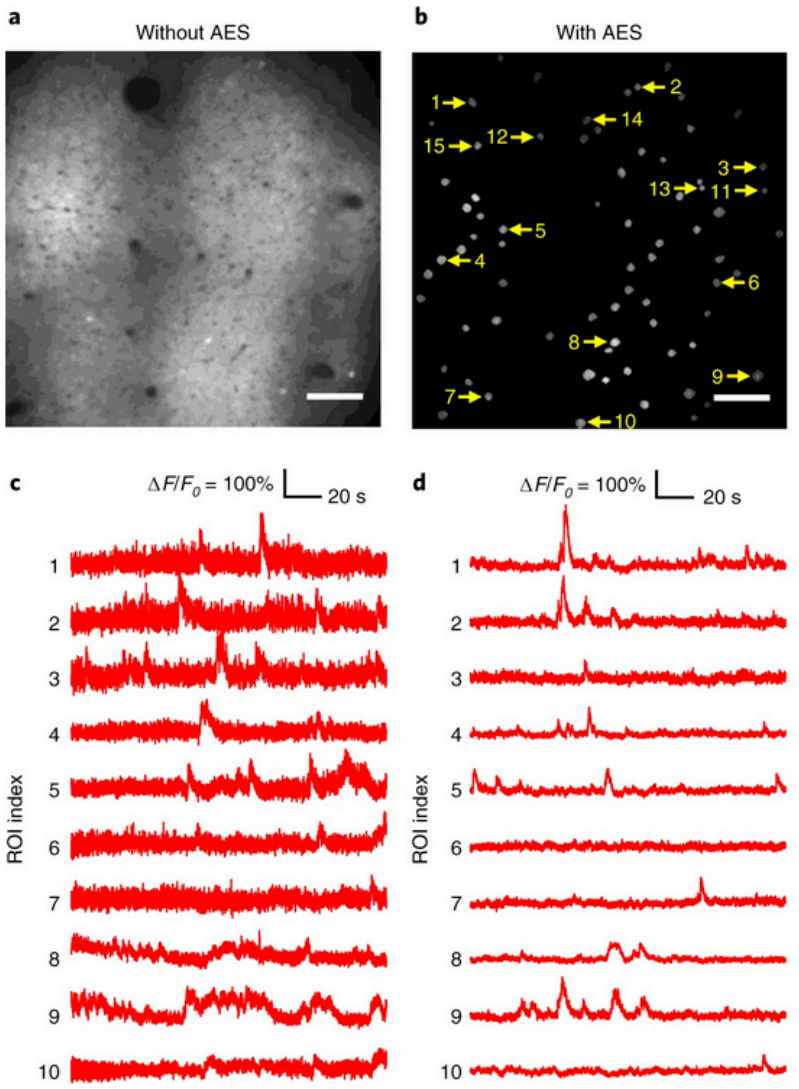
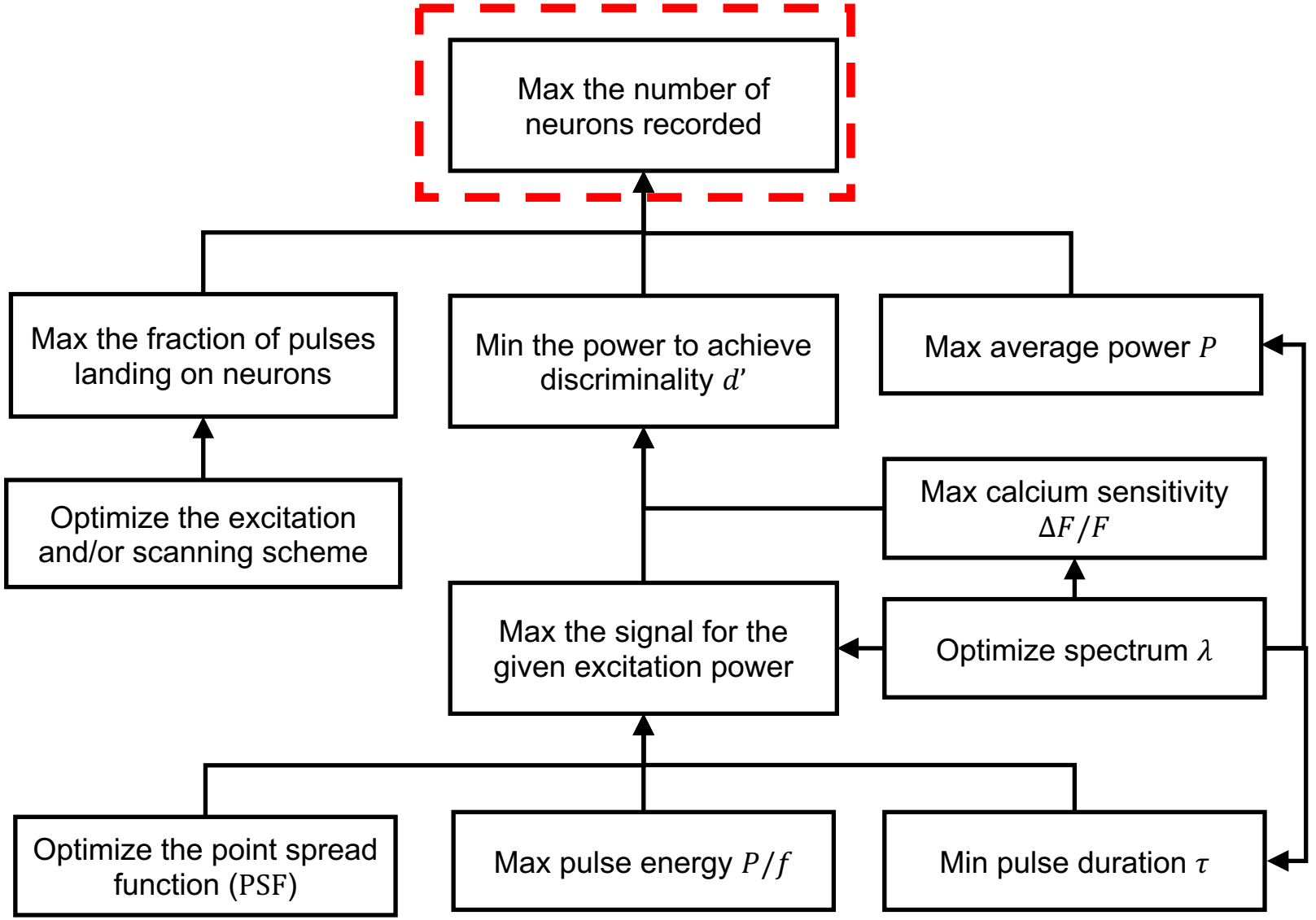
# Adaptive Excitation Source (AES)

- AES is a laser source that delivers excitation pulses only to the regions of interest (aka neurons).
- Such a scheme can dynamically change repetition rate to reduce average power by ~30x without sacrificing the signal.
- Meanwhile, the laser pulses are also amplified on demand, which allows a higher instantaneous repetition rate.




"An adaptive excitation source for high-speed multiphoton microscopy" *Nat. Comm.* (B. Li .. C. Xu 2020) https://doi.org/10.1038/s41592-019-0663-9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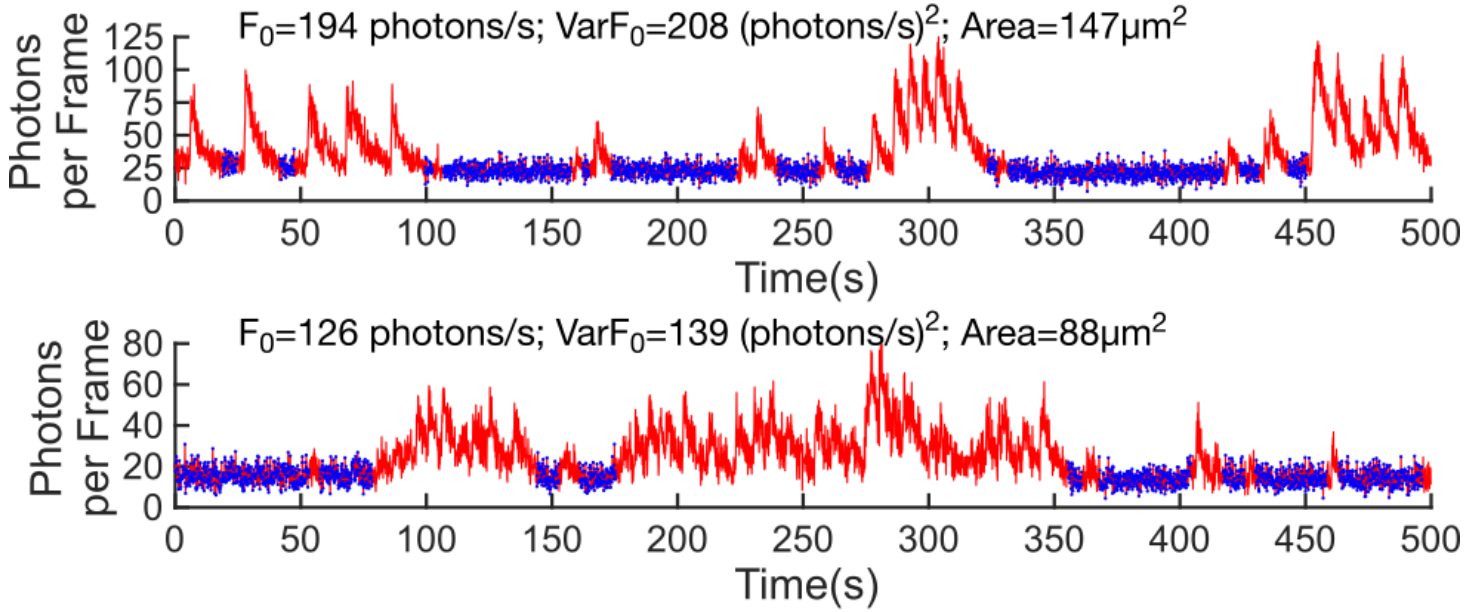
# The Minimum Signal Strength Required for Spike Inference

Instead of maximizing $d'$, we can find a minimum $d'$ required for $Ca^{2+}$ Imaging.
For $d' = 3, F_0 = 100$ photons per second is required for GCaMP6s.
For a burst of N spikes, their amplitude approximately add linearly, and 100/N photons are needed to detect their existence.

$$d' \approx \frac{\Delta F}{F}\sqrt{\frac{F_0 \tau}{2}}$$



# Estimation on the Maximum Number of Neurons Recorded by 3PM

- Find the optimal repetition rate at a given depth to maximize the total amount of 3PE signal.
- 3PE signal = photons generated by each pulse × repetition rate
- The photon budget for each neuron can also be calculated based on $d$' (e.g., 100 photons per second for $d' = 3$).
- The max number of neurons recorded = max 3PE signal / photon budget for each neuron.

For example: at 600 μm in cortex (~2 EAL), the optimal repetition rate is ~ 7 MHz; 3PE signal = (0.1 photons per pulse) × (7 MHz) = $7x10^5$ photons/s;

The max number of neurons recorded = ($7x10^5$ photons/s) / (100 photons/s) = 7000

# Challenges for Mesoscopic 3PM Imaging

To achieve the full promise of 3PE imaging, the following technical challenges need to be addressed:

- Realization of excitation sources around 1300 nm with energetic pulses (~μJ), on-demand pulse delivery, and high instantaneous repetition rate (10s MHz).
- Scanning scheme that allows immediate optical access (~ms) to thousands of neurons in ~1 × 1 × 0.5 $mm^3$ volume in 3D space.

The best solutions to these problems are still open!

For more technical review and prospect discussion on 3PM volumetric imaging, please refer to our review paper recently published in *Optica*.

"Three-photon neuronal imaging in deep mouse brain " *Optica* (T. Wang and C. Xu, 2020) https://doi.org/10.1364/OPTICA.395825

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NKT Photonics (Denmark)
Calmar Lasers
Newport

**NIH**
**NINDS (BRAIN)**
**NIBIB**
**NEI (BRAIN)**
**NIGMS**

**DARPA**

**IARPA MICrONS**

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