3 photon imaging of the mouse brain through the intact skull

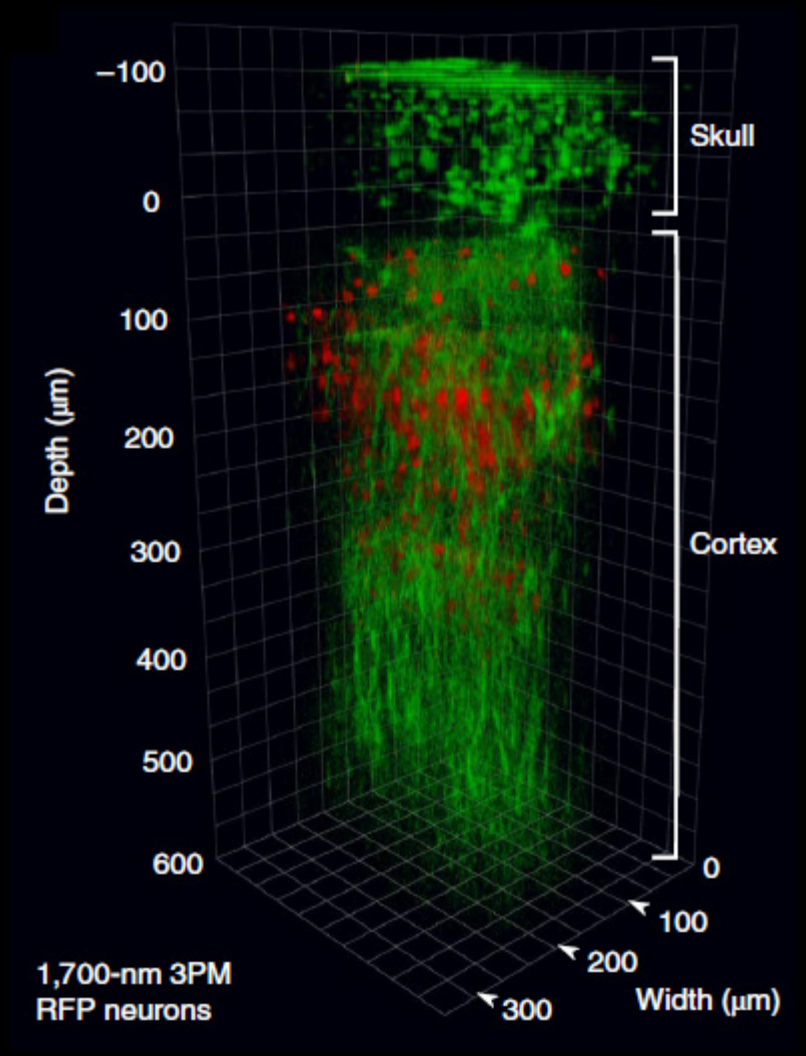


Image: Wang et el., Nat Methods. 2018 Oct;15(10):789-792.

Technical Journal Club
09.10.2018
Orsolya Török

Imaging techniques used for visualizing the brain

Imaging the brain *in vivo* with high integrity in its native enviroment is crucial to study:

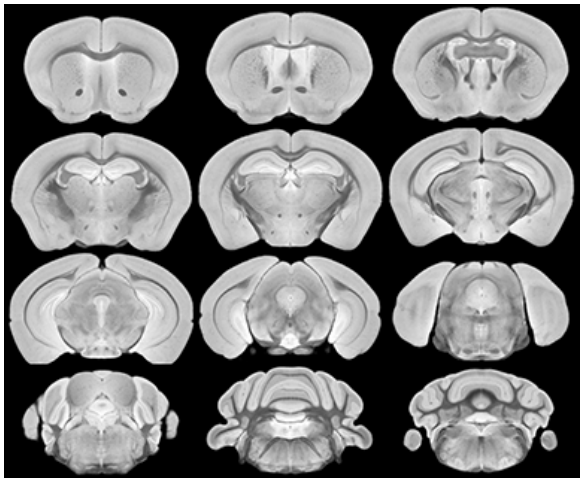
- neural network function
- cerebral blood circulation
- disease progression
- etc.

Methods used for imaging:

1. Non-fluorescent based technologies
2. One-photon fluorescent imaging
3. Two-photon microscopy
4. Three-photon microscopy

Non-fluorescence based imaging techniques

1. X-ray computed tomography (CT)
2. Magnetic resonance imaging (MRI)
3. Photoacustic tomography
4. Optical microangiography



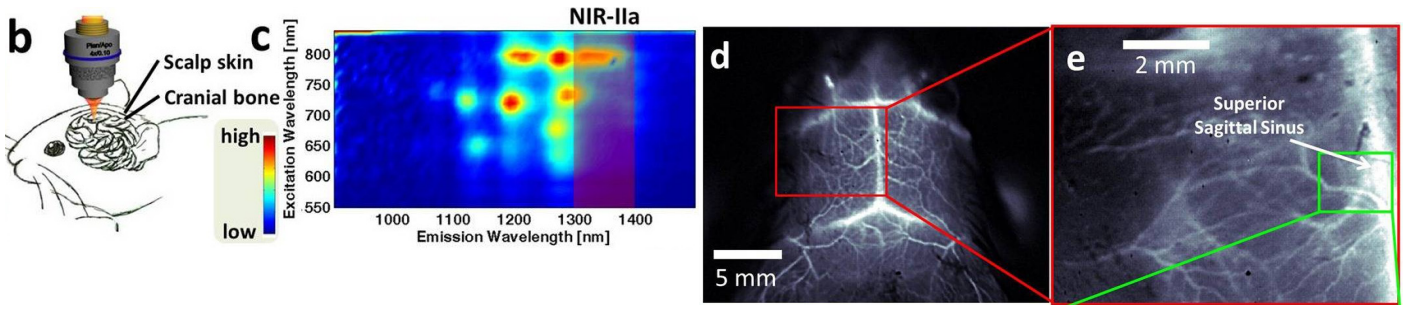
www.AARNet.edu.au

- routinely used
- deep tissue penetration – whole brain imaging
- spatial resolution is low: can not achieve single-cell or subcellular resolution, none of them is suitable direct cellular activity measurements with high temporal resolution (>1 Hz)

One-photon fluorescent imaging

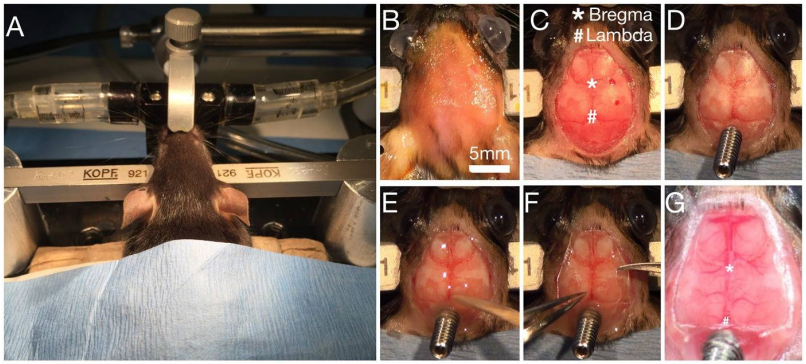
In combination with:

1. infrared dyes



Hong et al., Through-skull fluorescence imaging of the brain in a new near-infrared window; Nat Photonics. 2014 Sep;8(9):723-730.

2. skull-clearing methods (skull thining or cranial window implantation)

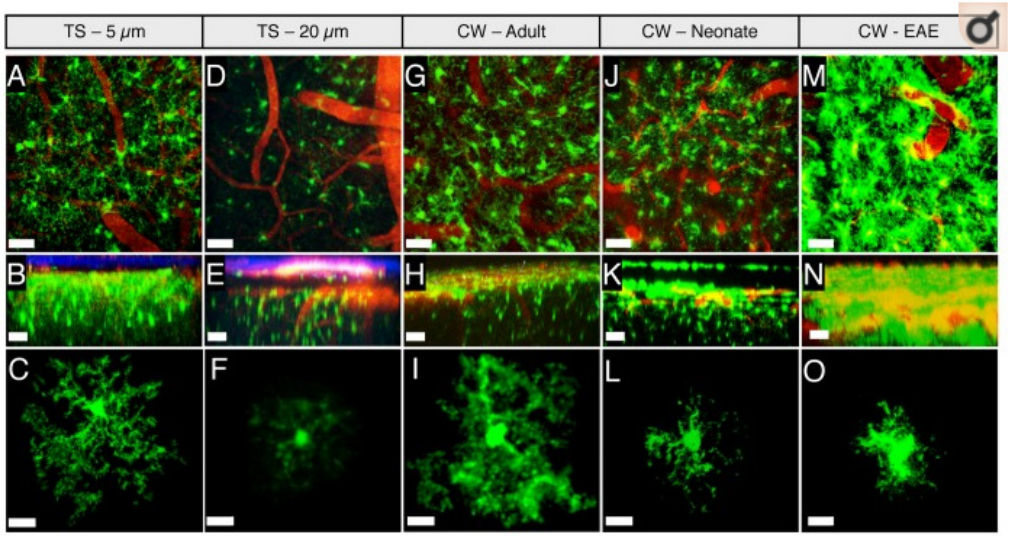


Silasi et al., Intact skull chronic windows for mesoscopic wide-field imaging in awake mice; J Neurosci Methods. 2016 Jul 15;267:141-9.

- Can be used to image:
  - vasculature
  - neuronal activity
- Do not provide:
  - single cell resolution <- out of focus fluorescent light

Two-photon miroscopy

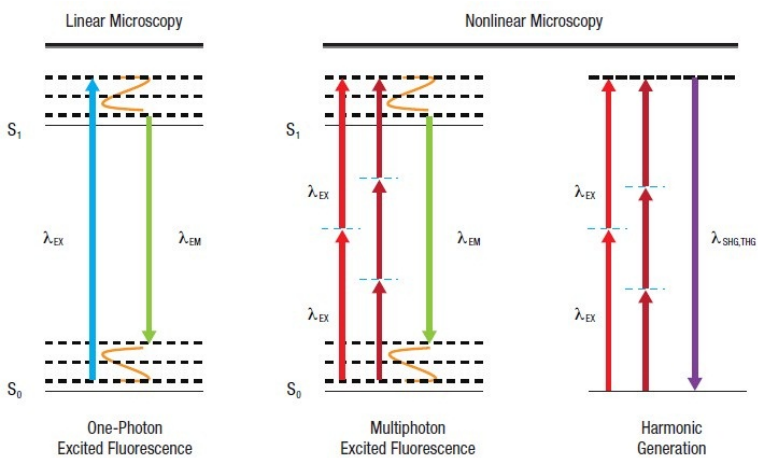
- Routinely used for *in vivo imaging*, because of its favourable tissue penetration depth
- Achieved by: *fs* excitation laser with a wavelength in the near-infrared (NIR) region (700-1000 nm) -> less photon absorption in biological tissues
- *Limitation:* very high photon scattering propeties of the skull -> skull-thining techniques or craniotomy + cranial window implantaion is necessary
- These interventions might cause:
    - Mechanical stress -> activation of microglia and astrocytes at acute imaging
    - Craniotomy -> change intracranial pressure or affect fluid flow in the paravascular space

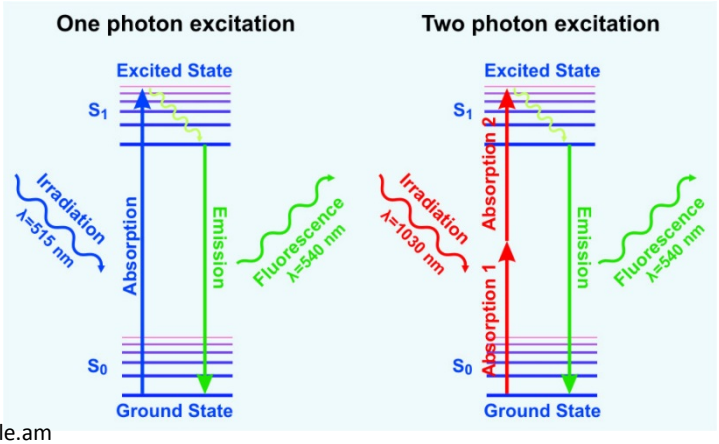


Dorand et al., Comparison of intravital thinned skull and cranial window approaches to study CNS immunobiology in the mouse cortex; Intravital. 2014 May;3(2).

Three-photon miroscopy

- Due to the higher order non-linear excitation and longer excitation wavelength (NIR-II: 1000-1700 nm) -> results in:
  - less scattering , enables imaging through the skull
  - much less out of focus fluorescence
- Limitation of the available fluorophores:
  - small 3 photon absorption
  - potential toxicity
  - irregular blinking of quantum dots (QDs)
  - photothermal damage
  - aggregation caused quenching





ACS NANO

Cite This: ACS Nano 2017, 11, 10452-10461

www.acsnano.org

Aggregation-Induced Emission Luminogen with Deep-Red Emission for Through-Skull Three-Photon Fluorescence Imaging of Mouse

Yalun Wang,[†,⊥] Ming Chen,[‡,⊥] Nuernisha Alifu,[†] Shiwu Li,[§] Wei Qin,[‡] Anjun Qin,[*,§] Ben Zhong Tang,[*,‡] and Jun Qian[*,†]

[†]State Key Laboratory of Modern Optical Instrumentations, Center for Optical and Electromagnetic Research, Joint Research Laboratory of Optics of Zhejiang Normal University and Zhejiang University, Zhejiang University, Hangzhou 310058, China

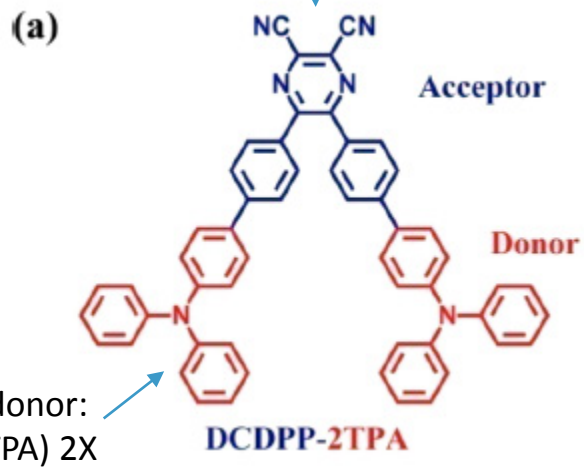
[‡]Department of Chemistry, Hong Kong Branch of Chinese National Engineering Research Center for Tissue Restoration and Reconstruction, Division of Life Science, State Key Laboratory of Molecular Neuroscience, Institute for Advanced Study, Institute of Molecular Functional Materials, Division of Biomedical Engineering, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

[§]Guangdong Innovative Research Team State Key Laboratory of Luminescent Materials and Devices, South China University of Technology, Guangzhou 510640, China

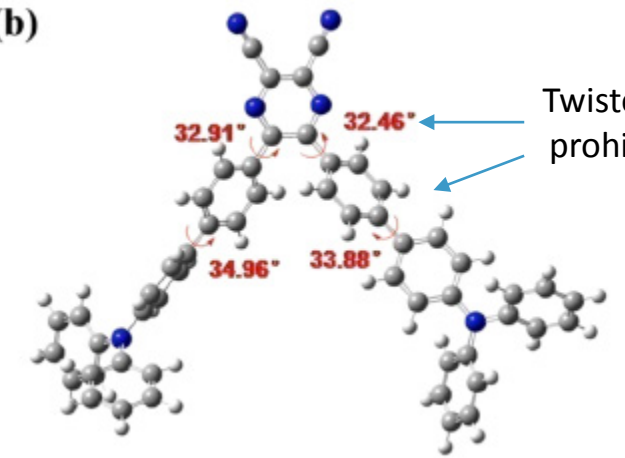
Aim: developement of a new aggregation-induced emission (AIE) luminogen dye with deep-red emission for 3 photon imaging

Molecular structure and aggregating feature of the new dye

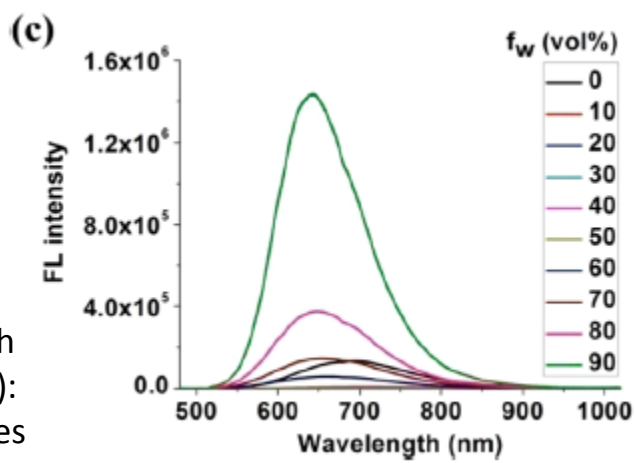
Strong electron-acceptor:
DCDPP (AIE luminogen)

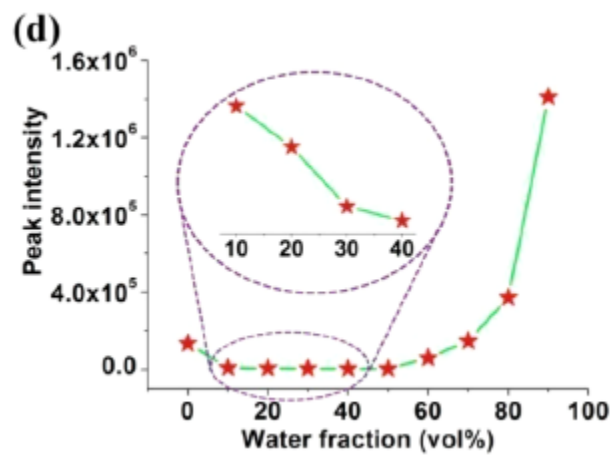


Strong electron-donor:
triphenylamine (TPA) 2X



Twisted conformation:
prohibits π-π stacking

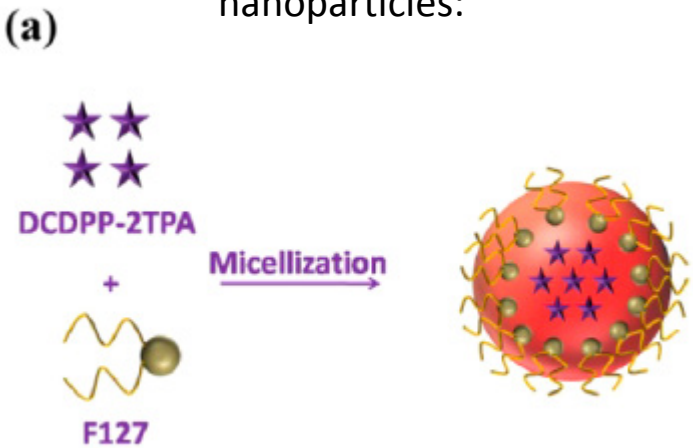




In mixtures with
high f_w(50-90%):
Larger aggregates

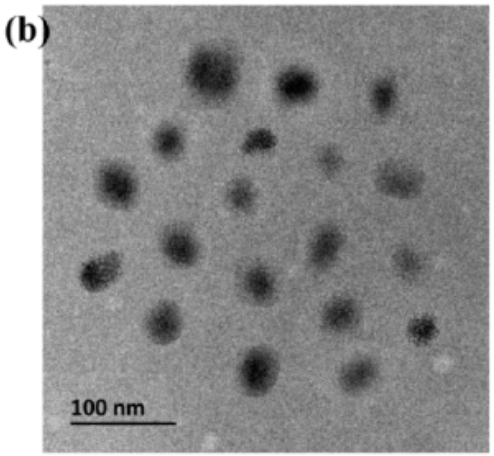
Preparation and characterization of DCDPP-2TPA

Aqueously dispersable nanoparticles:

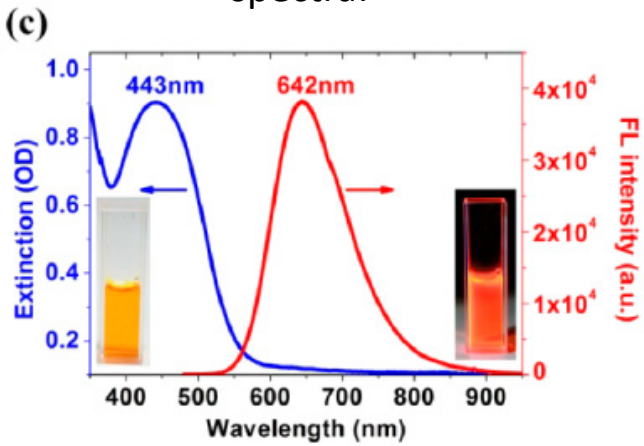


Visualization with TEM

d_{avg}=29 nm → Suitable for the ciculation in blood vessels



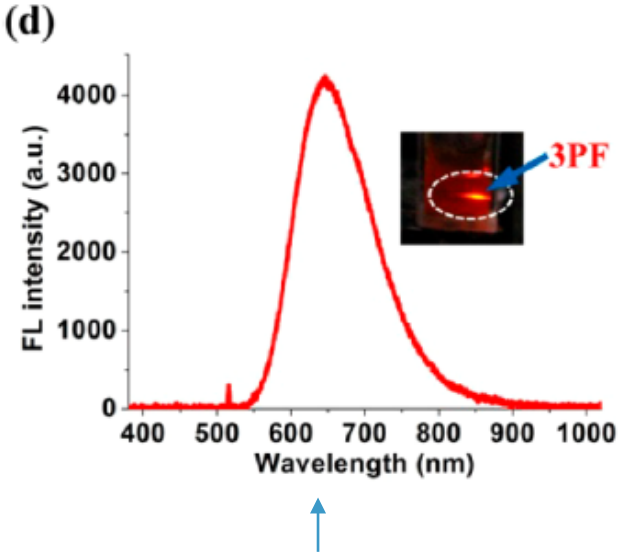
Excitation and emission spectra:



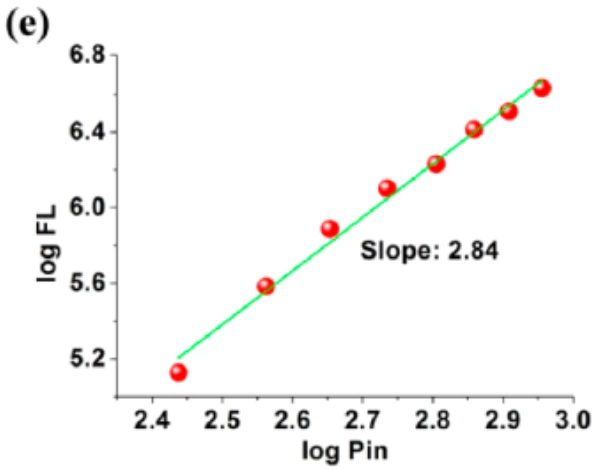
→ Bright deep-red emission

3-photon fluorescent properties of DCDPP-2TPA

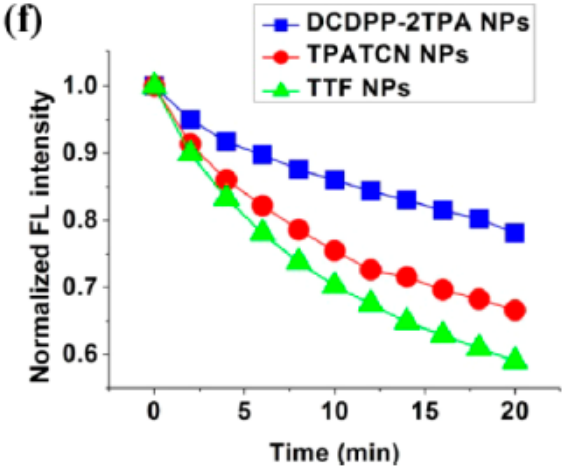
Excitation with 1550 nm laser



Excitation power dependence

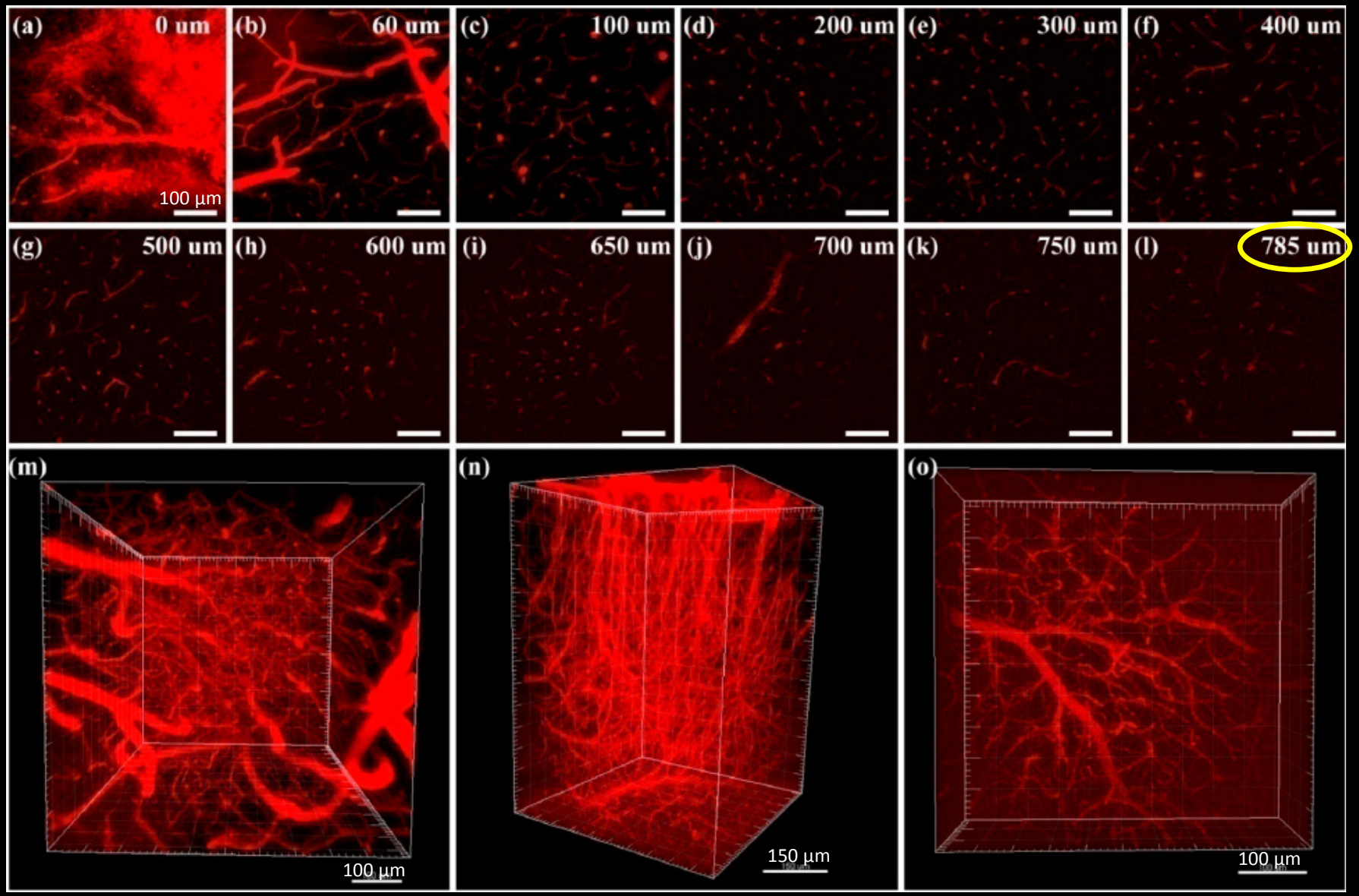


Photostability



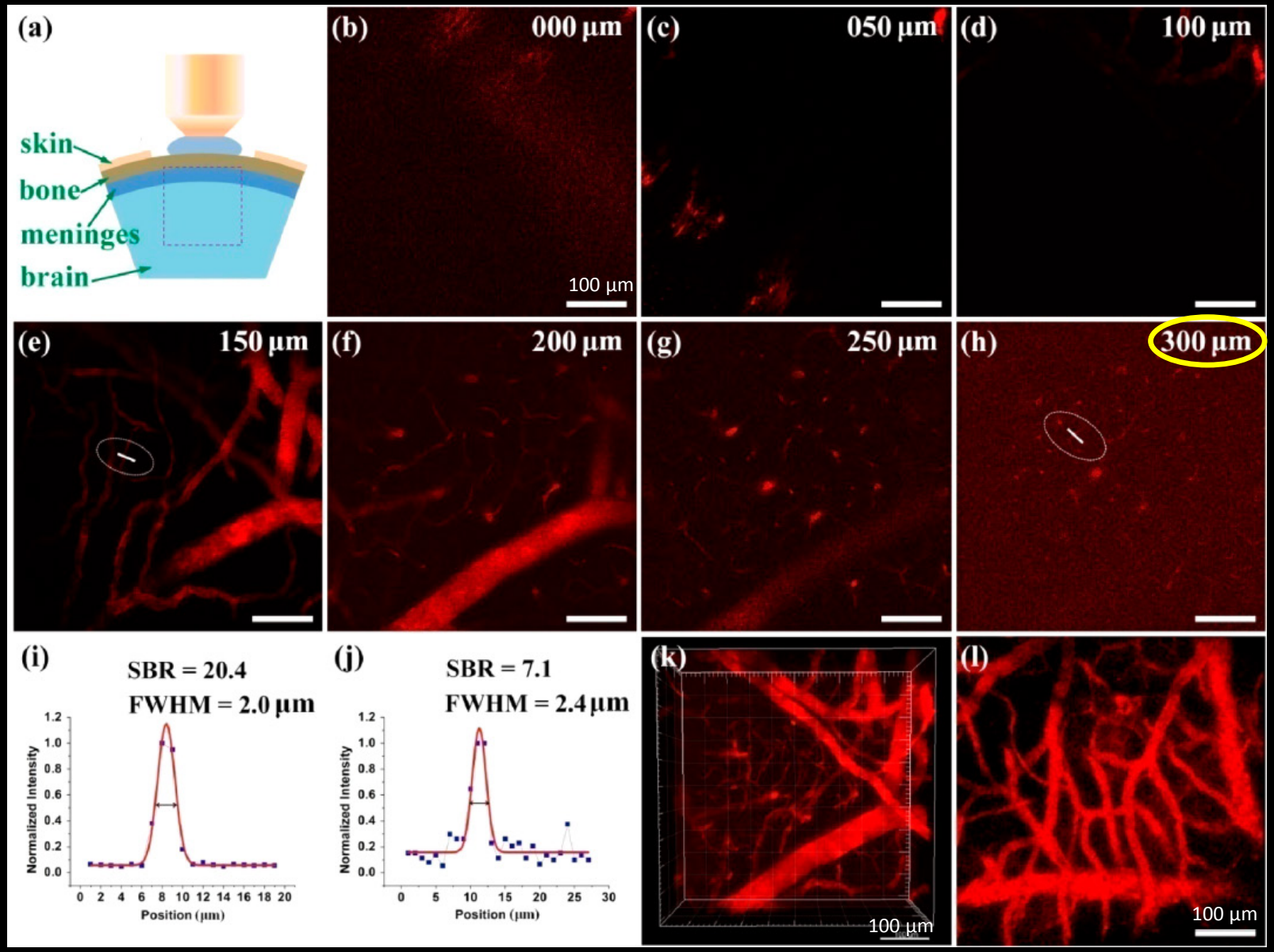
In vivo 3-photon fluorescent microscopic imaging with opened skull

Excitation with 1550 nm laser



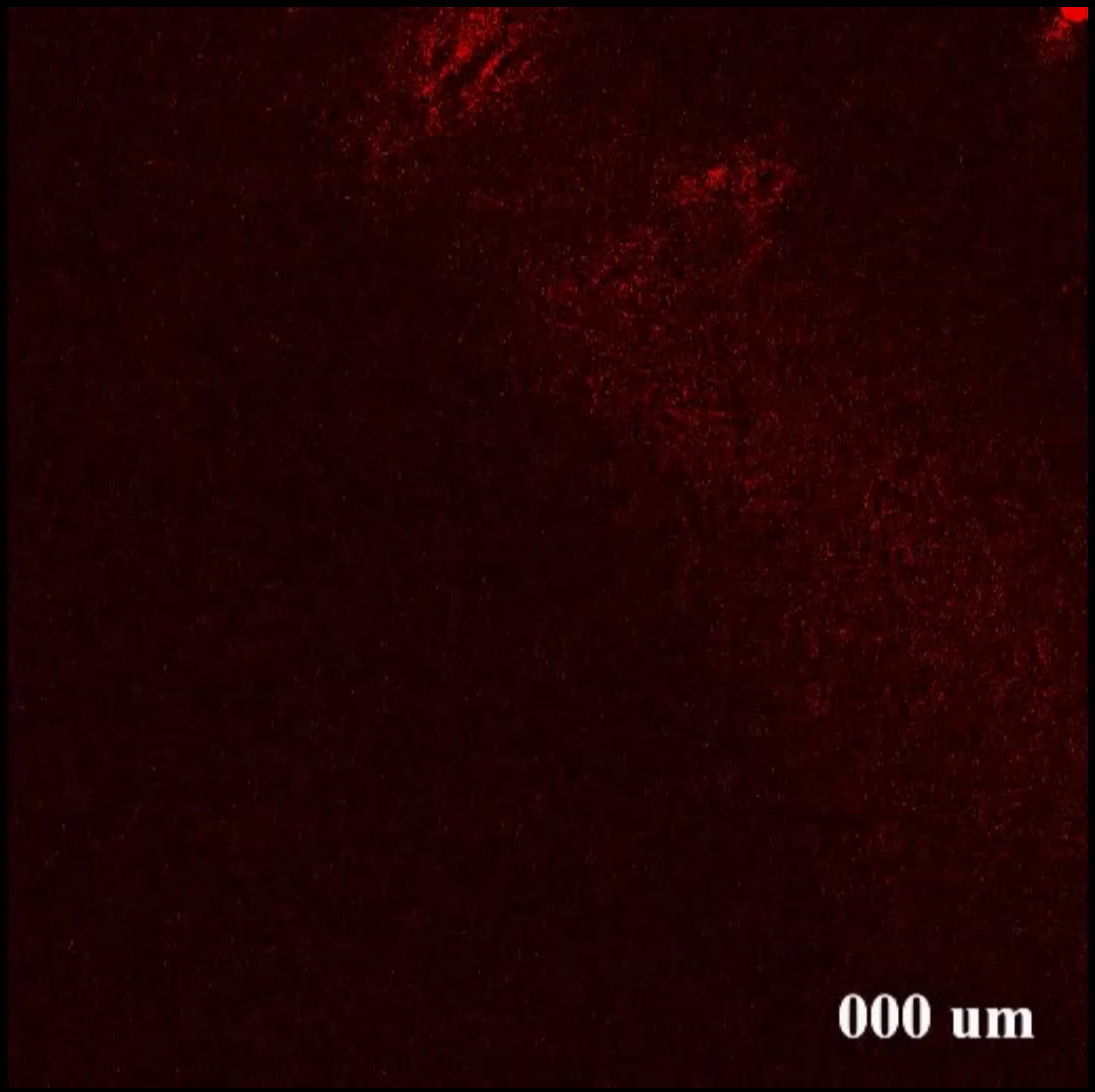
In vivo 3-photon fluorescent microscopic imaging with intact skull

Excitation with 1550 nm laser



In vivo 3-photon fluorescent microscopic imaging with intact skull

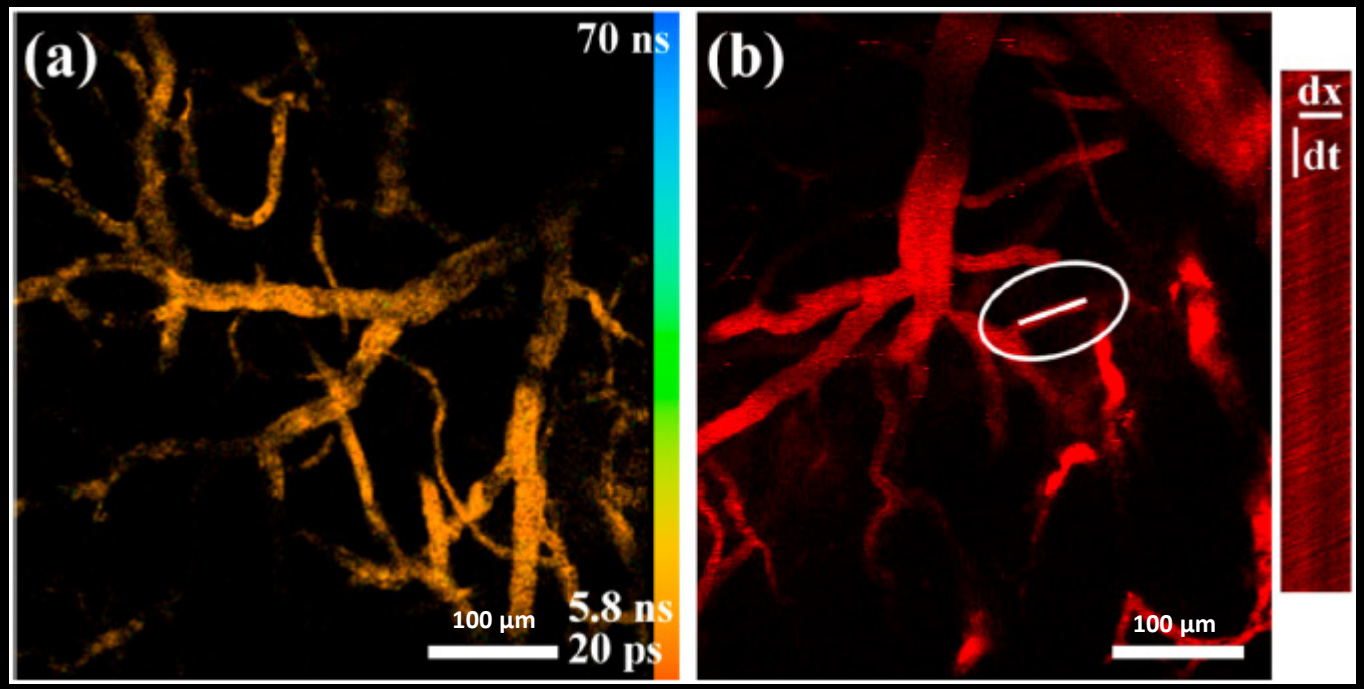
Excitation with 1550 nm laser



In vivo 3-photon fluorescent microscopic imaging with intact skull

Excitation with 1550 nm laser

In vivo lifetime imaging ***Blood flow velocity***



Velocity= ~2.4 mm/s

Conclusion

- Improvements:
  - an AIE luminogen dye was synthetized
  - in form of aqueously dispersible nano particles
  - under the excitation with 1550 nm fs laser had a peak 3-photon fluorescence emmision at ~650 nm
  - with good photostability
  - imaging through an intact skull was performed
  - tissue penetration depth of 300 µm
  - capillaries as small as 2.4 µm were resolvable
  - suitable for blood flow measurements

- Limitations:
  - not commercially available dye
  - costly imaging
  - imaging only of the vasculature possible

nature methods

BRIEF COMMUNICATION

https://doi.org/10.1038/s41592-018-0115-y

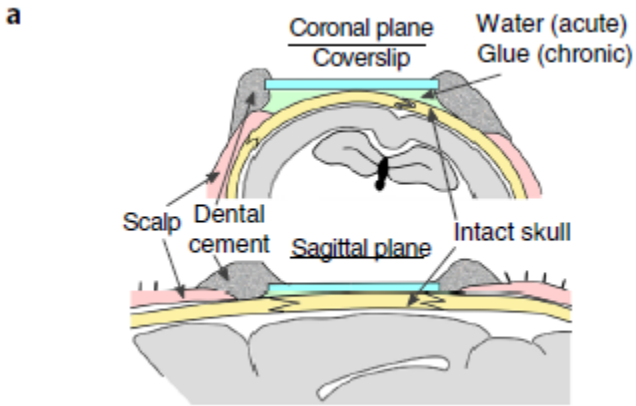
Three-photon imaging of mouse brain structure and function through the intact skull

Tianyu Wang[1*], Dimitre G. Ouzounov[1], Chunyan Wu[1], Nicholas G. Horton[1], Bin Zhang[2], Cheng-Hsun Wu[2], Yanping Zhang[2,3], Mark J. Schnitzer[2,3] and Chris Xu[1*]

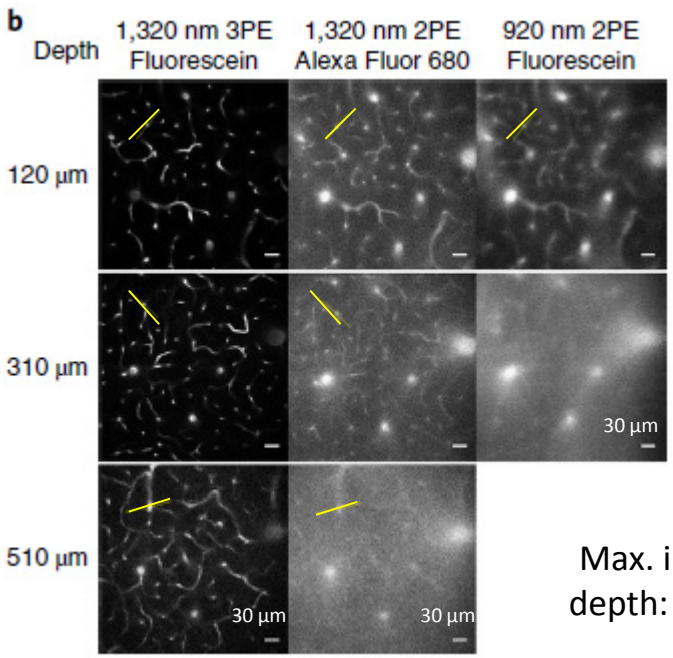
[1]School of Applied and Engineering Physics, Cornell University, Ithaca, NY, USA. [2]CNC Program, Stanford University, Stanford, CA, USA. [3]Howard Hughes Medical Institute, Stanford University, Stanford, CA, USA. *e-mail: tw329@cornell.edu; cx10@cornell.edu

Comparison of 2-photon microscopy and 3-photon microscopy through intact skull

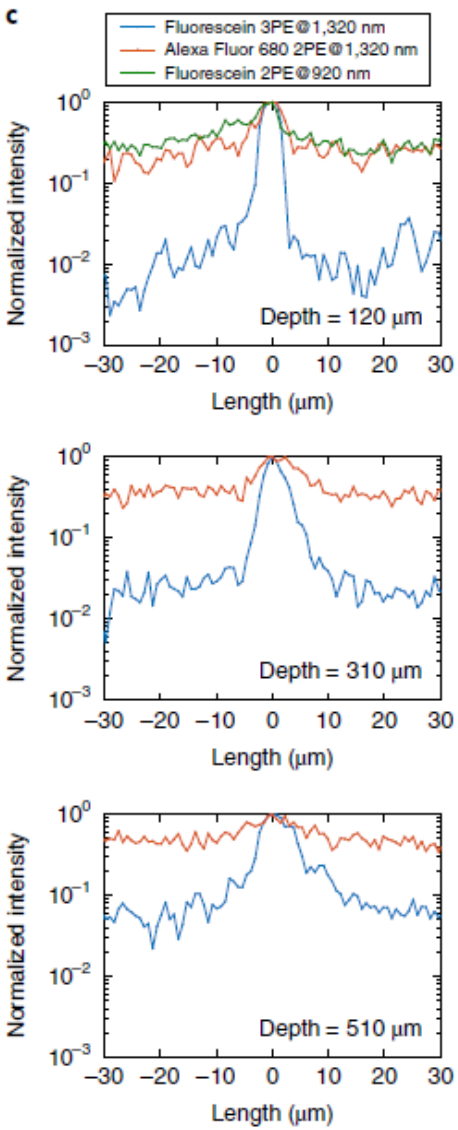
Imaging setup:



Comparison:

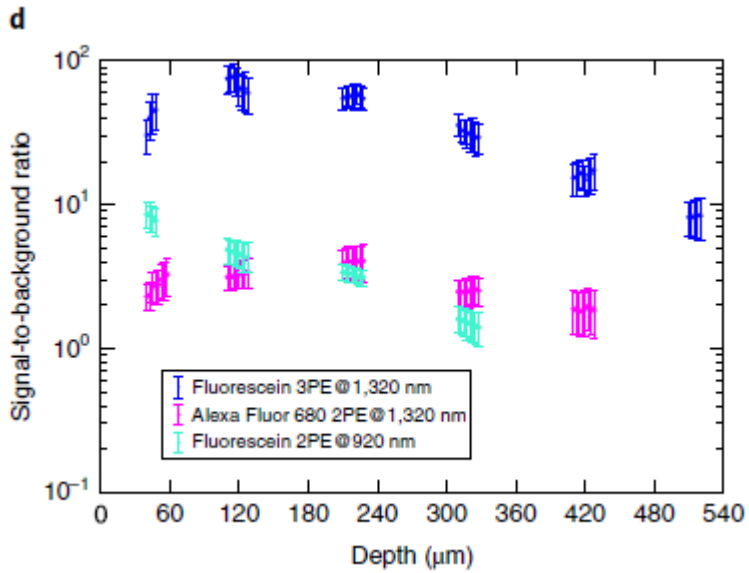


Max. imaging depth: 420 µm

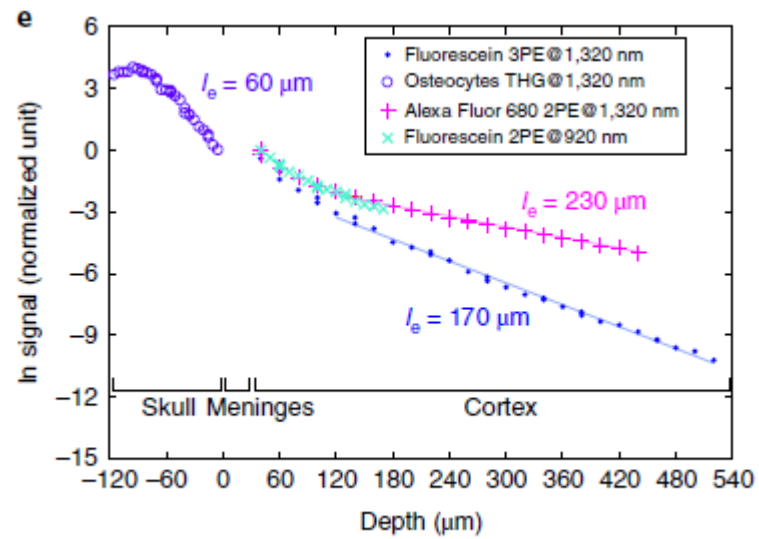


Comparison of 2-photon microscopy and 3-photon microscopy through intact skull

Signal to background ratio (SBR):



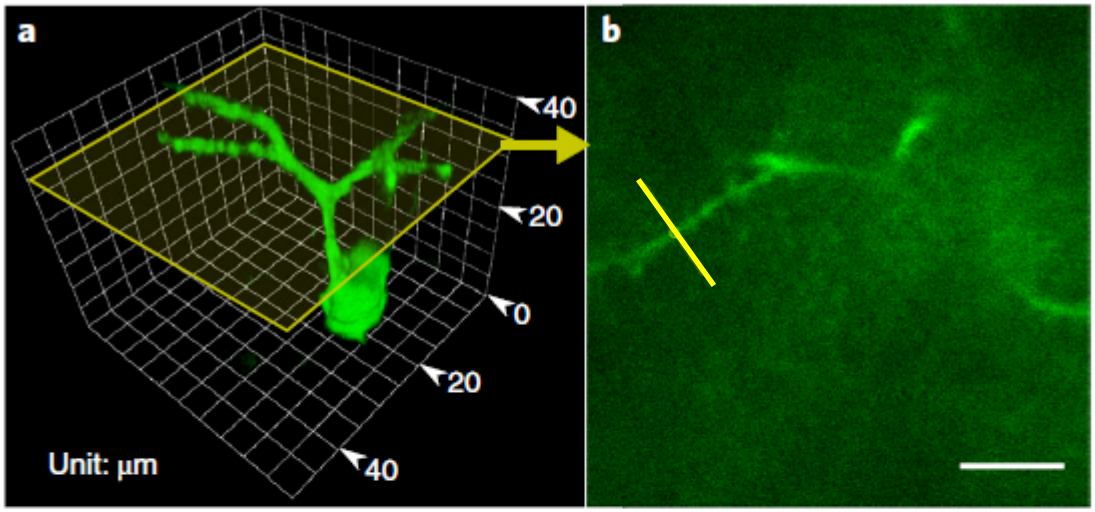
Signal attenuation with depth:

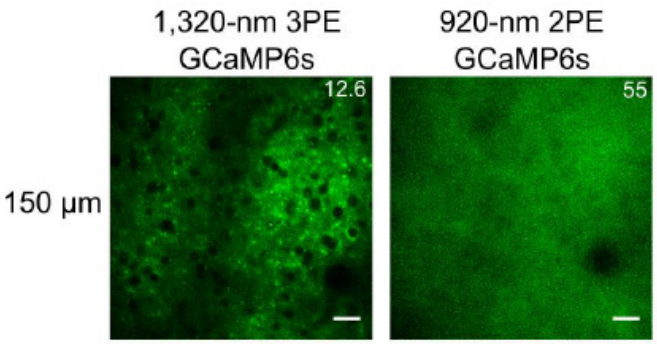


⇒ Concluded that longer excitation wavelength alone is not enough, 3-photon excitation is necessary for through-skull imaging

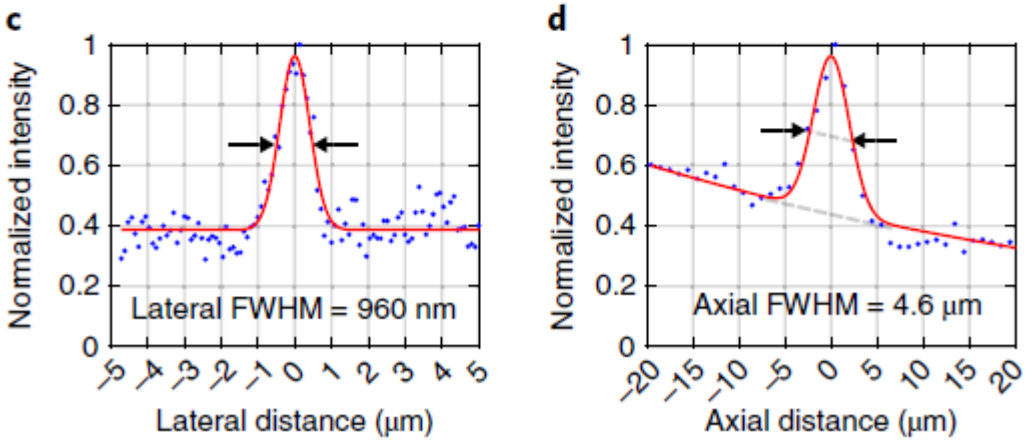
Assessment of spatial resolution

- GCaMP6s reporter mice
- excitation with 1320 nm laser





⇒ 920 nm 2-photon microscopy failed to resolve any feature at high spatial resolution

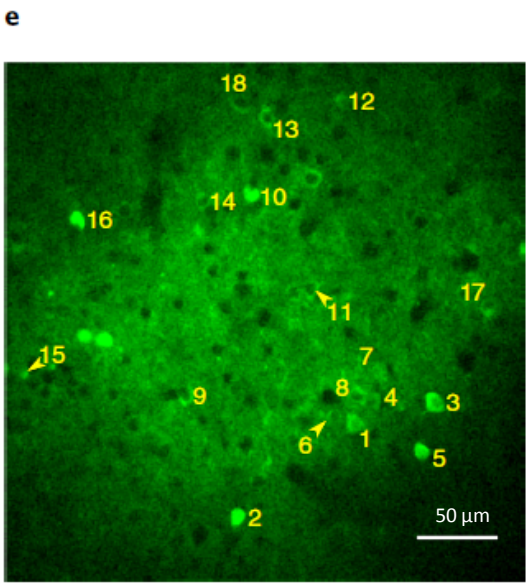


Max. imaging depth: 465 µm

Imaging of spontaneous activities

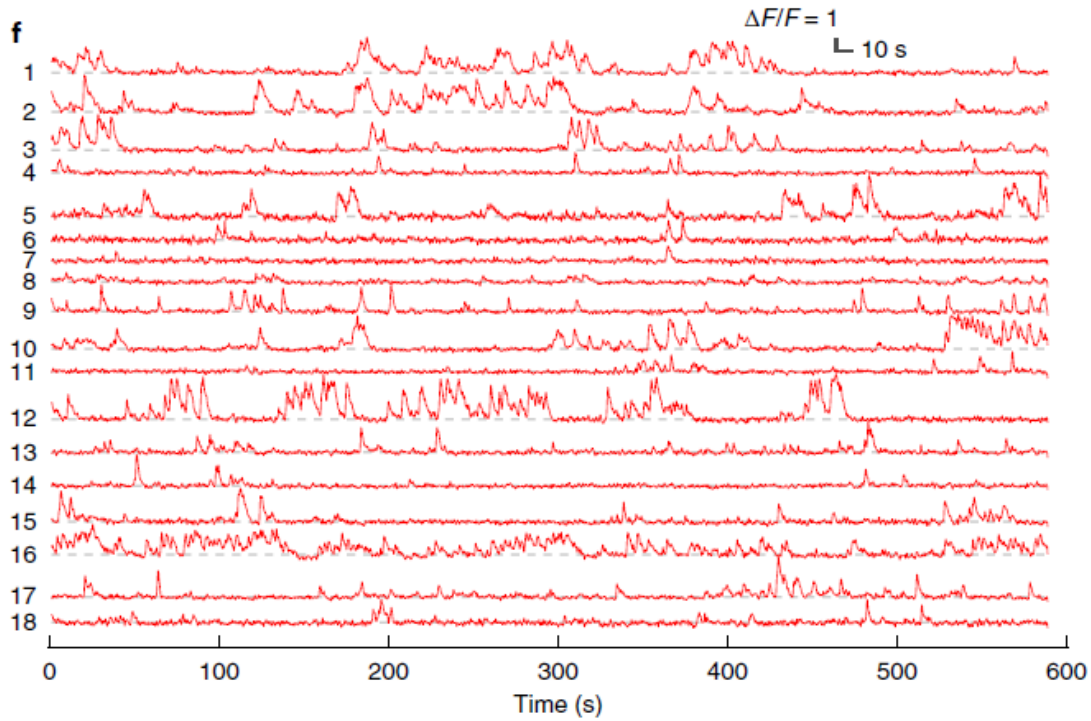
- GCaMP6s reporter mice
- excitation with 1320 nm laser

Imaging site in cortical layer 2/3



275 µm below the cortical surface

Recording of neuronal activity traces under awake conditions

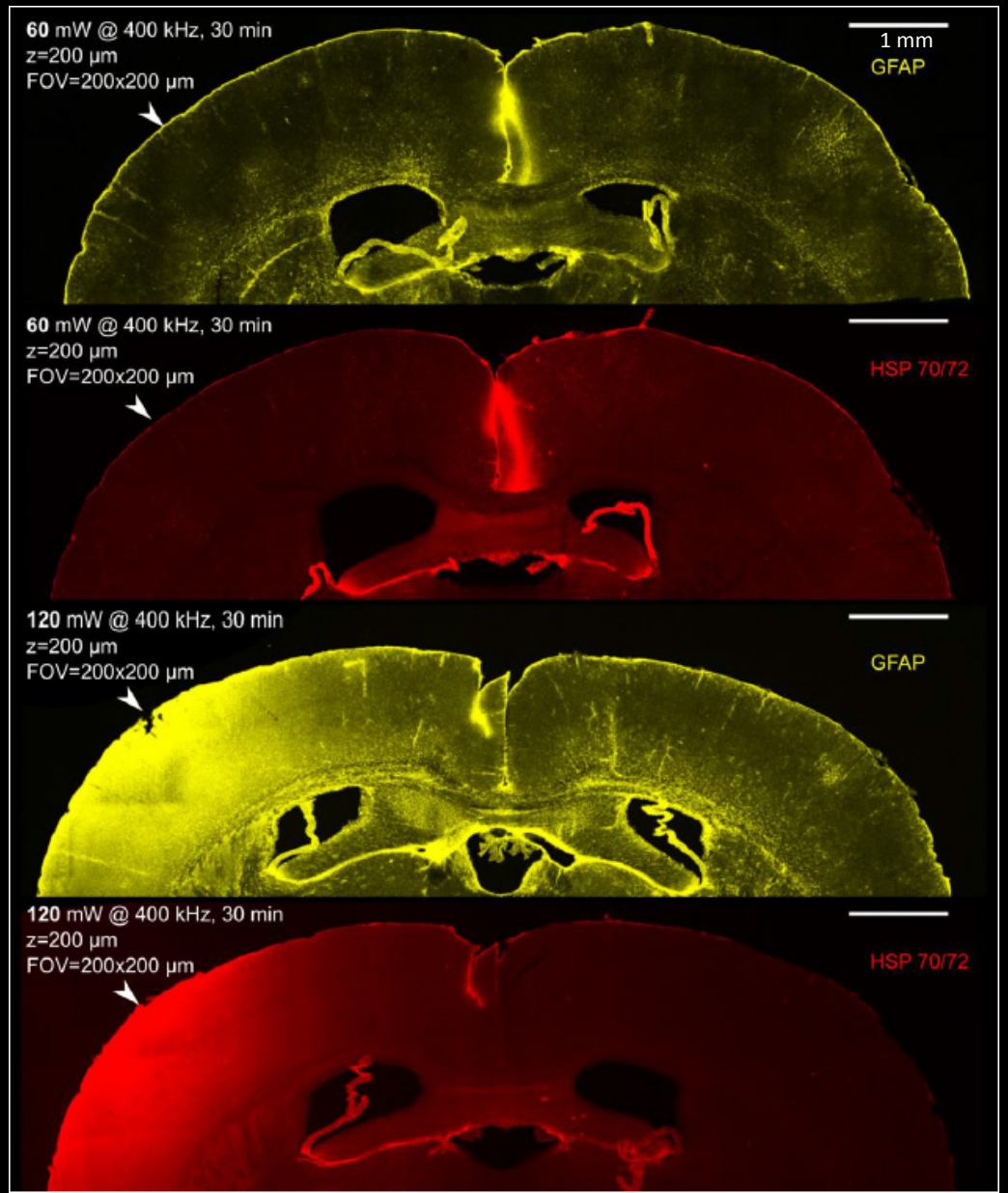


Absolute signal to noise ratio was calculated

⇒ results were comparable to the results obtained from 2-photon microscopy with cranial window

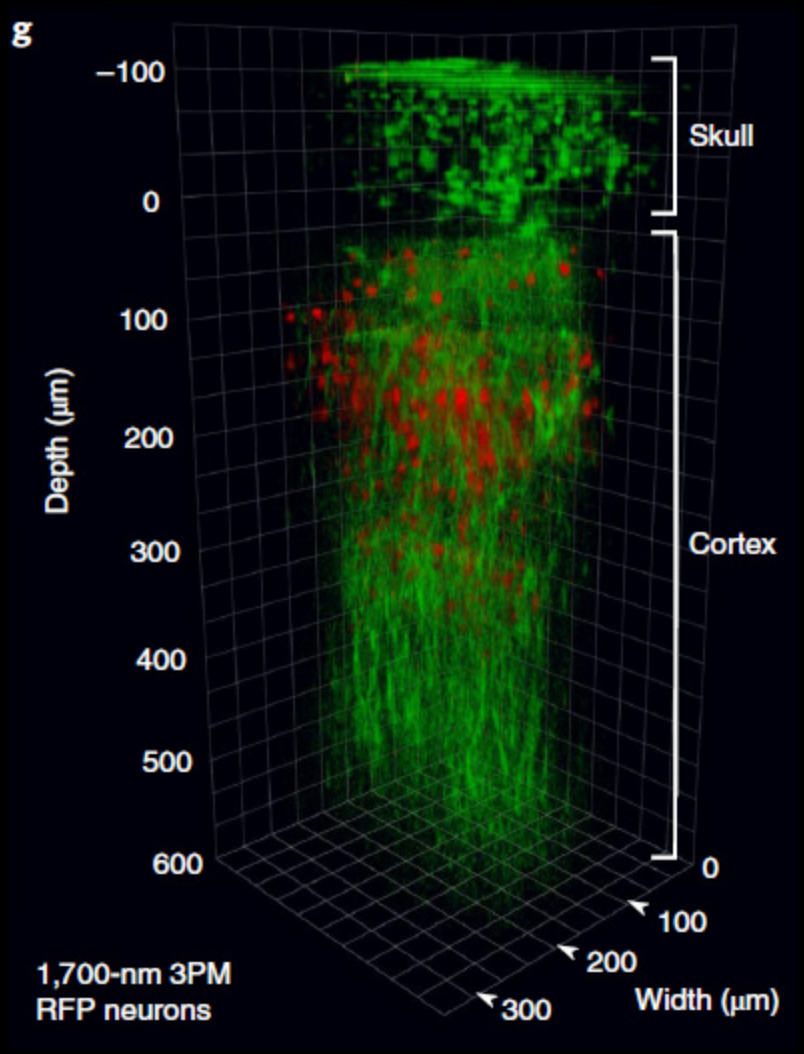
⇒ However, to maintain the SNR and temporal resolution, photon counts per neuron per second had to be increased -> limited its performance

Immunostainings to detect possible tissue damage due to high laser power



Testing of 1700 nm 3-photon excitation

- RFP-labelled neurons in Brainbow-mice
- THG signal is in green



Conclusion

- Improvements:
  - 3 photon-microscopic imaging through intact skull
  - with high spatial and temporal resolution
  - large field of view (FOV)
  - at a substantial depth (510 µm)

- Limitations:
  - In functional imaging to maintain the SNR and temporal resolution, laser power had to be increased -> limited its performance

Thank you for your attention!

Image: ZEISS Microscopy via Creative Commons