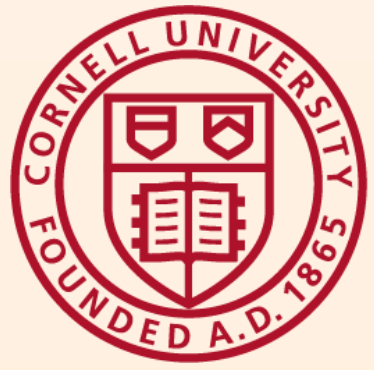




Multi-color three-photon fluorescence imaging with single-wavelength excitation deep in mouse brain



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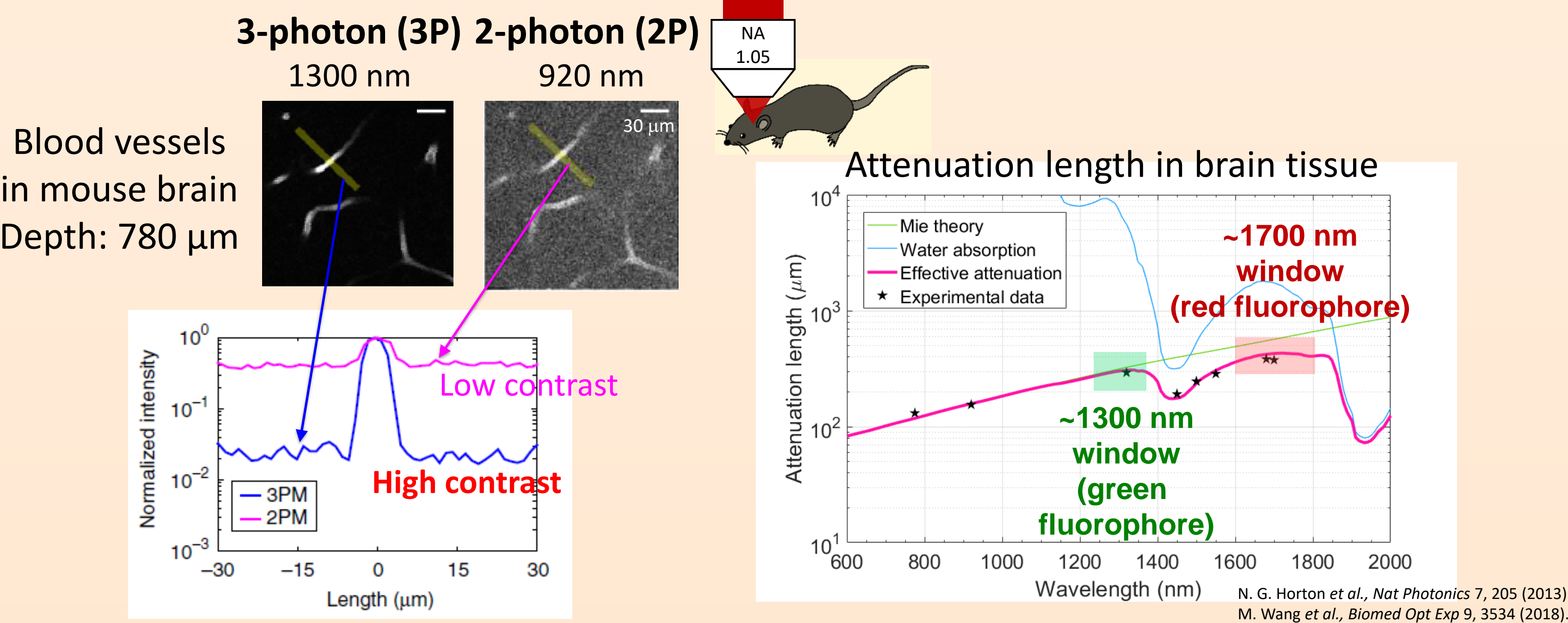
Summary

We proposed a new 3-photon excitation scheme: excitation to a higher energy state, which enables;

- ➔ Multi-color 3PM with single-wavelength excitation at ~ 1300 nm, using green and red fluorophores
- ➔ $\sim 10\times$ signal enhancement in some red fluorophores.

(Please find our paper for more details;
Y. Hontani, F. Xia, C. Xu, *Science Advances* 7, eabf3531 (2021).)

3-photon microscopy (3PM)



D. G. Ouzounov, T. Wang et al., *Nat. Methods* 14, 388 (2017).

3PM enables high-contrast imaging in deep tissue beyond the 2PM depth limit.

Two optimal excitation windows for 3PM with low tissue scattering and water absorption:
(i) ~ 1300 nm, used for green fluorophores
(ii) ~ 1700 nm, used for red fluorophores

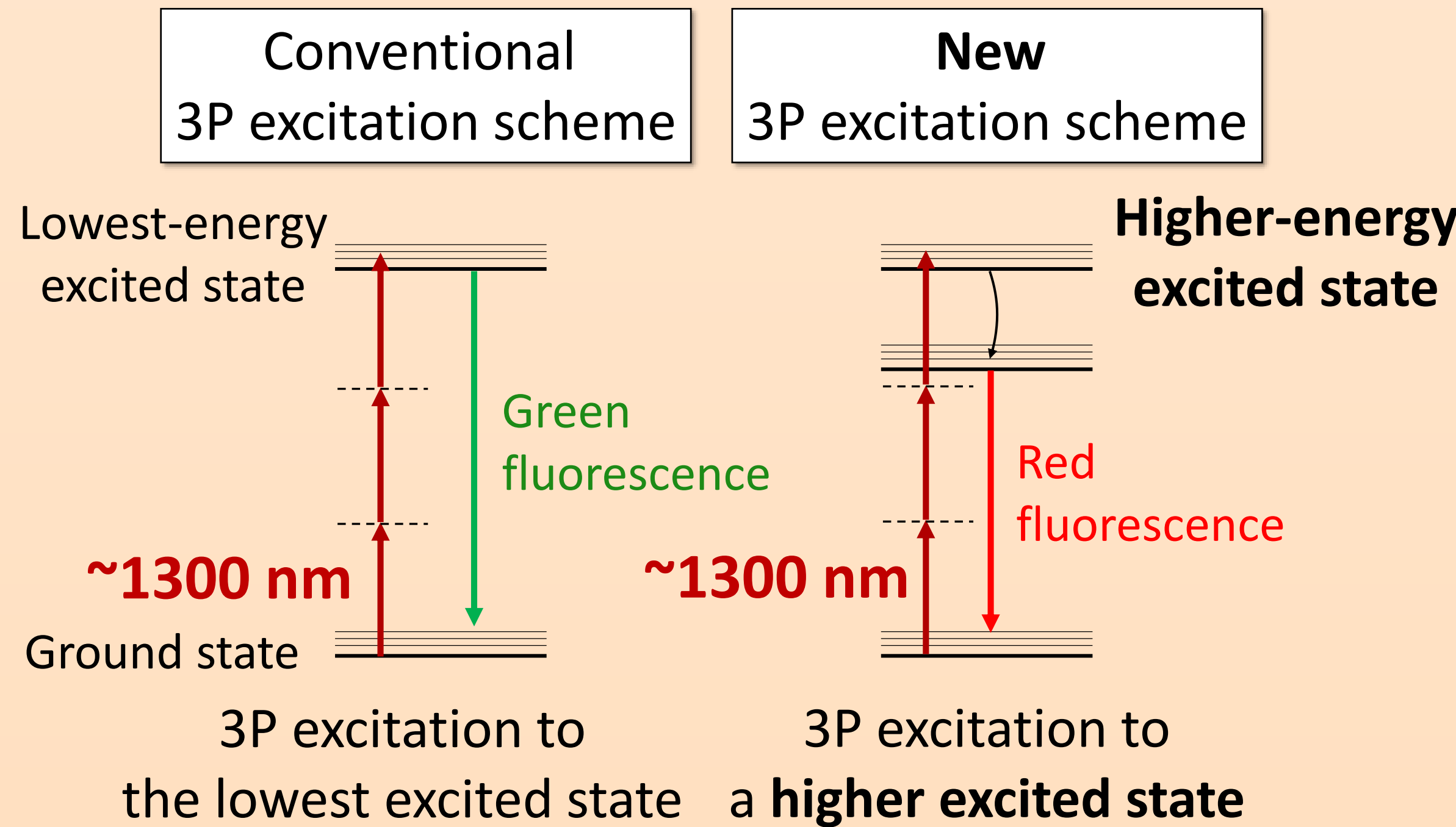
Challenges in 3PM

- (1) Multi-color 3PM with single wavelength excitation has been difficult.
because of the spectral separation for the optimal 3P excitation:
green fluorophores (~ 1300 nm) and red fluorophores (~ 1700 nm)
- (2) The 3P fluorescence intensity is weak.
($\sim 100\times$ weaker than 2P fluorescence with the same excitation pulse energy.)
because of the higher-order non-linear excitation process

This work

We address these two challenges in 3PM with a new 3P excitation scheme.

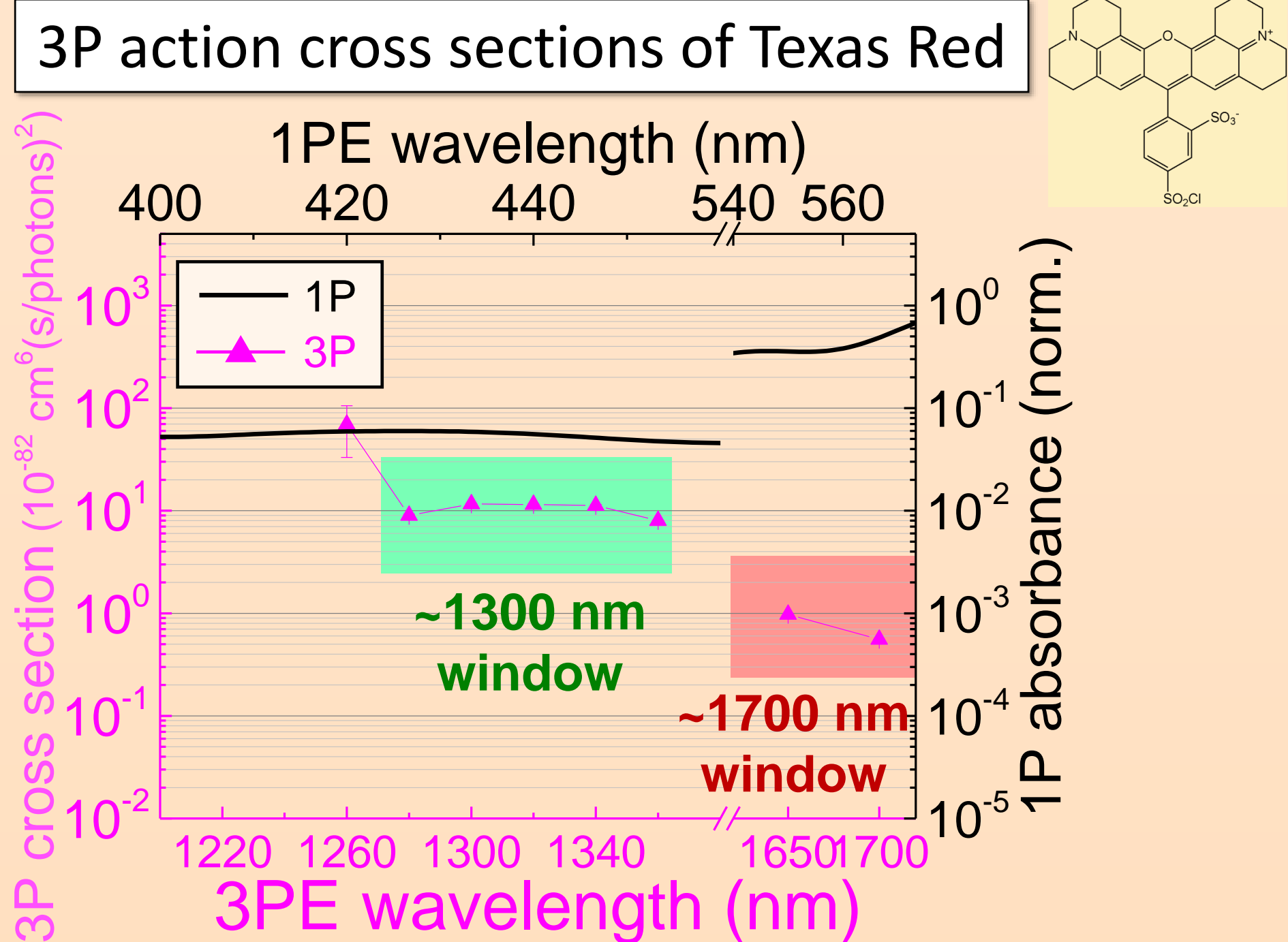
Approach



- With 3P excitation to a higher-energy excited state, a red fluorophore can be excited at ~ 1300 nm.
- Combining the new 3P excitation scheme (right) with the conventional scheme (left), both green and red fluorophores can be excited with single wavelength at ~ 1300 nm.

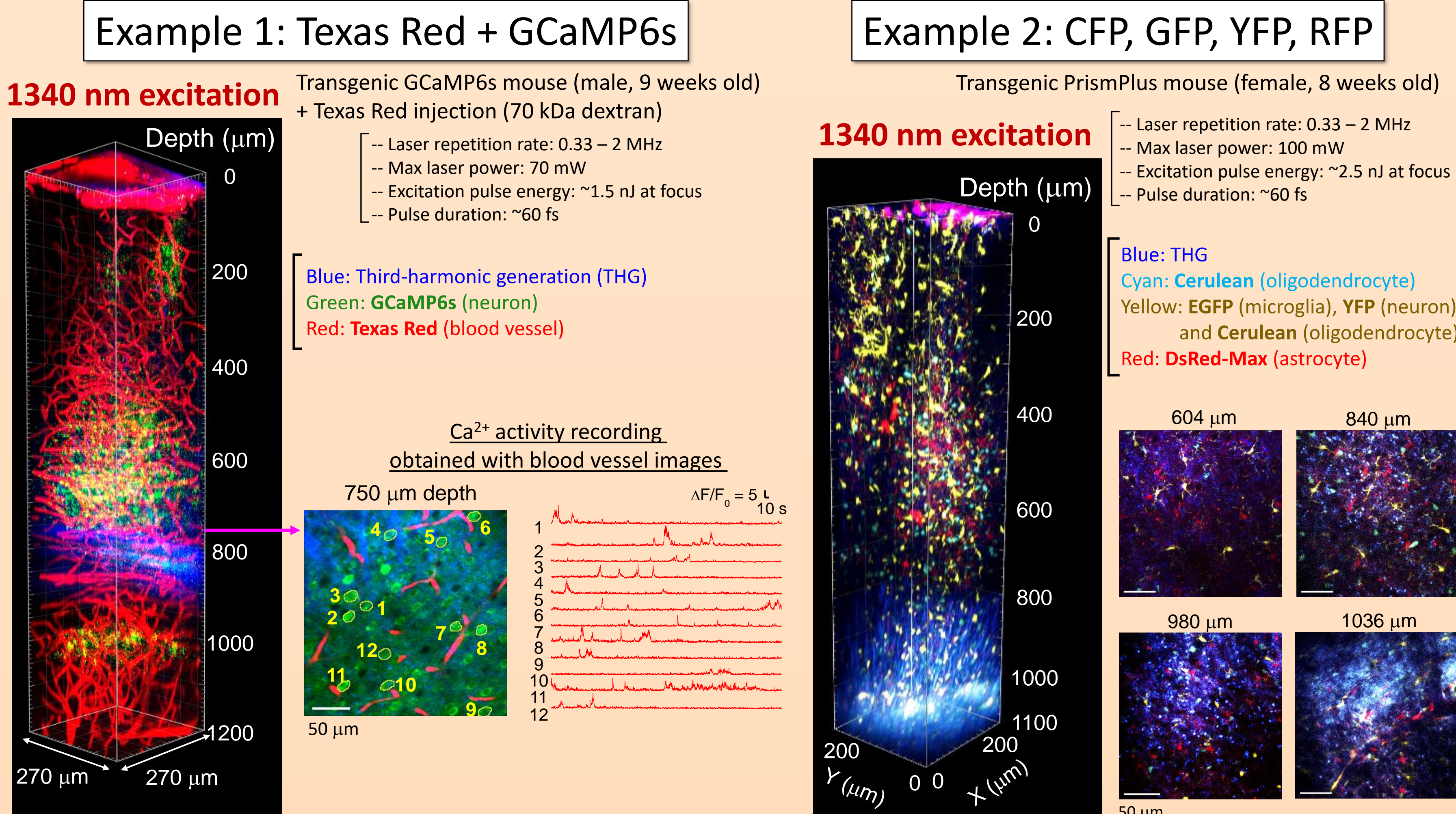
➔ It enables multi-color (green and red) 3PM with single wavelength at ~ 1300 nm.

3P action spectrum



- 3P excitation of Texas Red was detected at ~ 1300 nm (higher-excited state), in addition to ~ 1700 nm (lowest excited state).
- At ~ 1300 nm, $\sim 10\times$ stronger signal than at ~ 1700 nm was seen, likely due to the resonance enhancement effect.
- Adequately large 3P signals at ~ 1300 nm were also seen in other red fluorophores: SR 101, Alexa Fluor 546, DsRed, tdTomato, DsRed, and Qdot 605 (Data shown in the paper, Y. Hontani, F. Xia, C. Xu, *Science Advances* 7, eabf3531 (2021).).

Multi-color 3PM in living mouse brain



-- Simultaneous multi-color 3PM with 1340-nm excitation down to the CA1 hippocampus region was demonstrated, combining cyan/green/yellow fluorophores (excitation to the lowest excited state) with red fluorophores (excitation to a higher excited state).