Two-photon absorption cross-sections of eosin and hematoxylin

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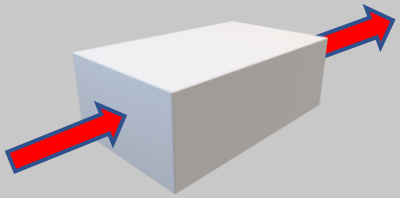
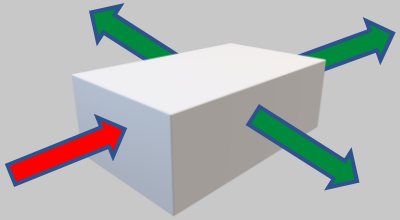
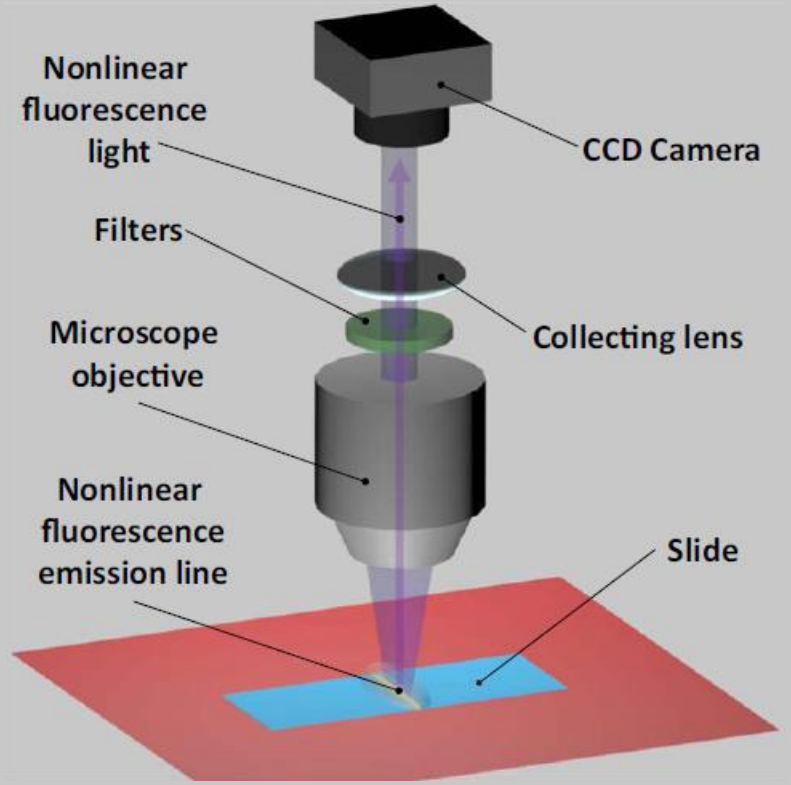
Measuring two-photon absorption

Direct methods	Indirect methods
Direct measurement of transmission losses originating from two-photon absorption (TPA)	Measurement of a quantity originating from a process subsequent to TPA, e.g. fluorescence
	
linear absorption, scattering	low signal level

Fluorescence generated by a line-shaped excitation



The linear excitation volume delivers a high photon flux even with a short confocal parameter.

➔ the signal level is high enough to avoid long integration times leading to noise

➔ a very accurate measurement of TPA spectra is thus possible.

Eosin & Hematoxylin

- frequently used in white-light microscopy

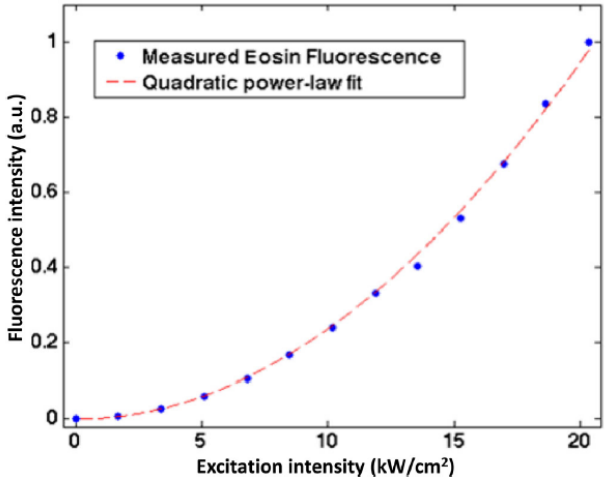
Advantages

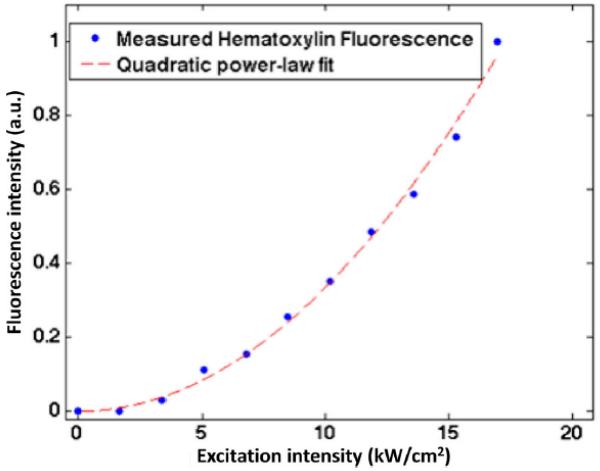
- accessible staining protocol
- feasible and relatively cheap staining
- quick and fine inspection of the tissue
- employed in several kinds of tissues
- very low photobleaching
- longtime staining persistence for storage
- now used in **one-photon** fluorescence microscopy ➔ generating a considerable quantity of fluorescence

Caveats

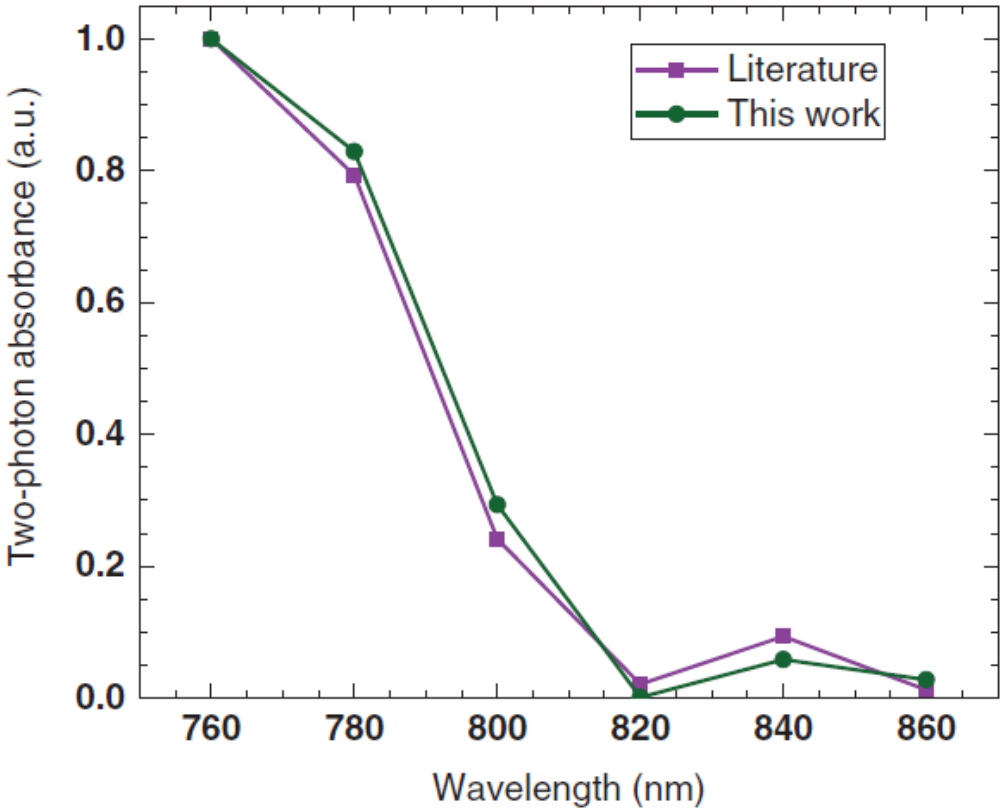
- need of finding the correct absolute and mutual concentration
- potential arising of inversion quenching phenomena

Calibration and validation





Alexa 488 dye



Experimental estimation

- relative measurement calibrated with reference sample

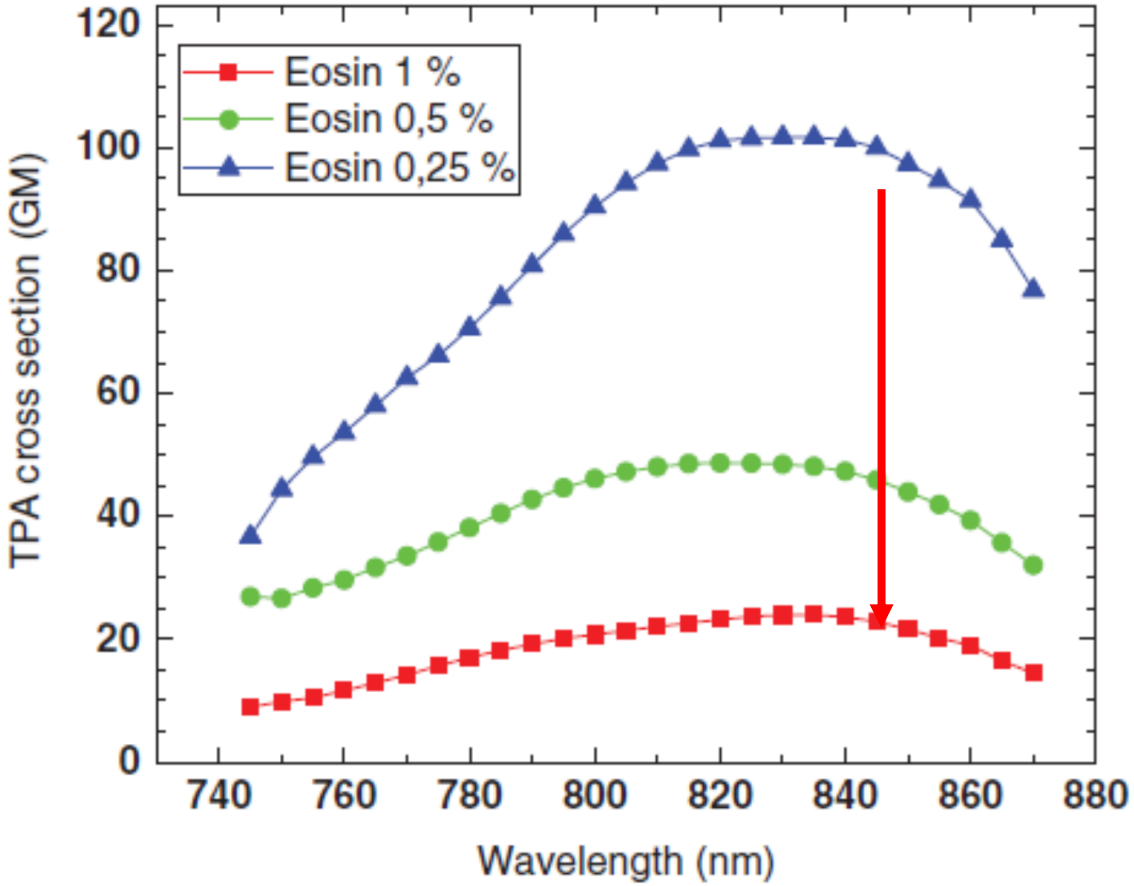
- known quantum yield ➔ cross section

$$\delta_E = \delta_A \frac{\phi_A \eta_A C_A}{\phi_E \eta_E C_E} \frac{F_E}{F_A},$$

REFERENCE (Alexa 488)
δ_A= cross section
φ_A= quantum yield
η_A= collection efficiency
C_A= concentration
F_A= fluorescence photons (per second)

SAMPLE (Eosin)
δ_E= cross section
φ_E= quantum yield
η_E= collection efficiency
C_E= concentration
F_E= fluorescence photons (per second)

Spectral two-photon cross section
Eosin



Dye	Concentrations (mM)
Eosin	14.45; 7.23; 3.61
Hematoxylin	3.31; 1.65; 0.83

➔ evident concentration quenching
(previosly observed @ lower concentrations)*

*A. Tuer et al., J. Biomed. Opt. (2010), **15**, 026018

Experimental estimation

- relative measurement calibrated with reference sample

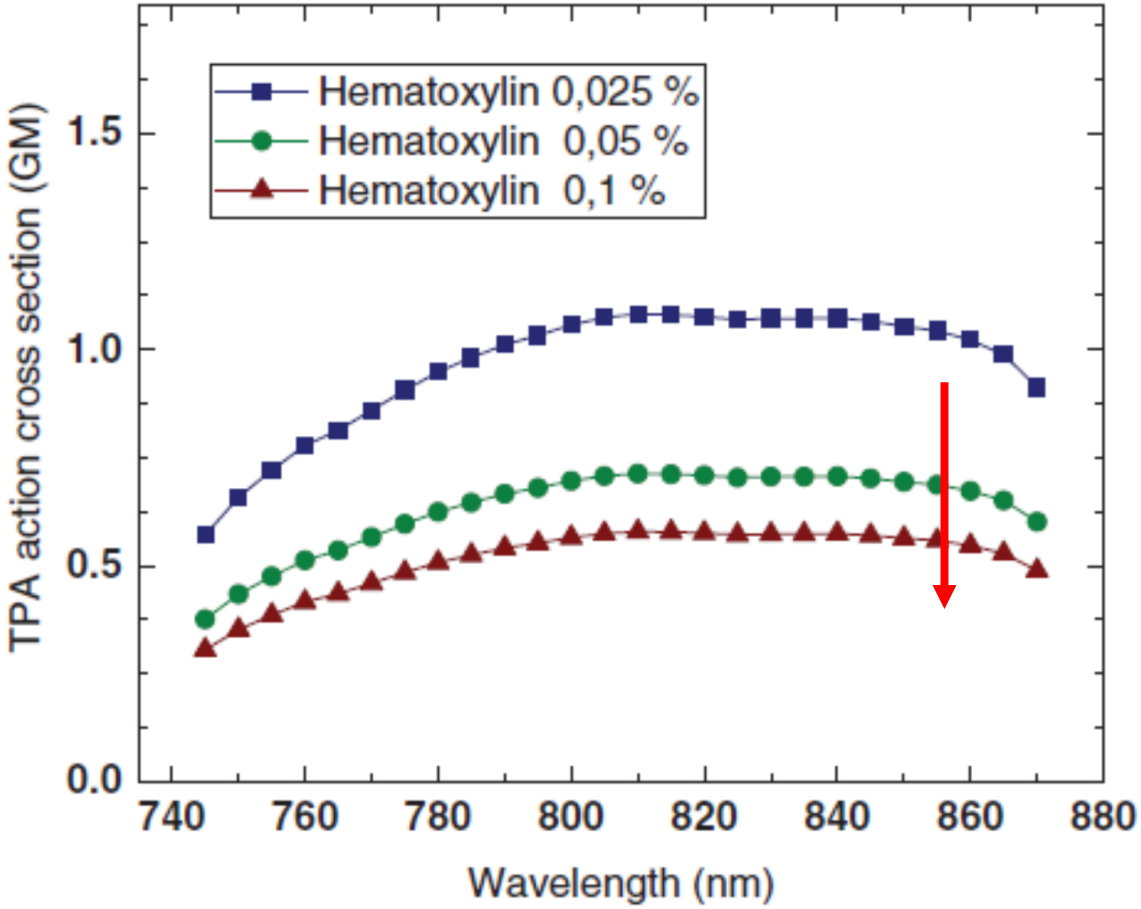
- unknown quantum yield ➔ action cross section

$$\delta_H^* = \delta_H \phi_H \rightarrow \boxed{\delta_H^* = \delta_A \frac{\phi_A \eta_A C_A}{\eta_H C_H} \frac{F_H}{F_A},}$$

REFERENCE (Alexa 488)
δ_A= cross section
φ_A= quantum yield
η_A= collection efficiency
C_A= concentration
F_A= fluorescence photons (per second)

SAMPLE (Hematoxylin)
δ_H= cross section
φ_H= quantum yield
$\delta_H = \delta_H \varphi_H$ = action cross section
η_H= collection efficiency
C_H= concentration
F_H= fluorescence photons (per second)

Spectral two-photon cross section hematoxylin



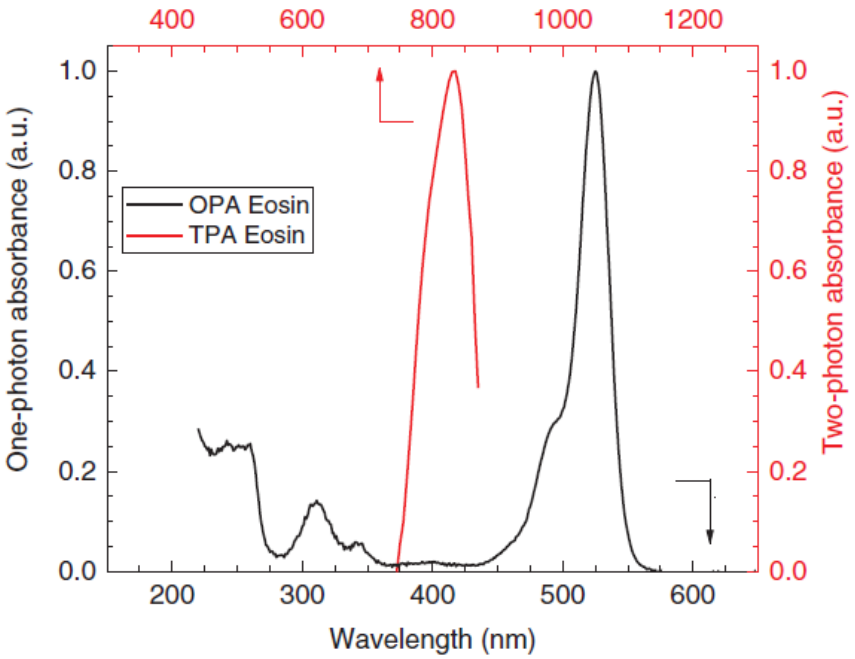
➔ confirmed 2-P features

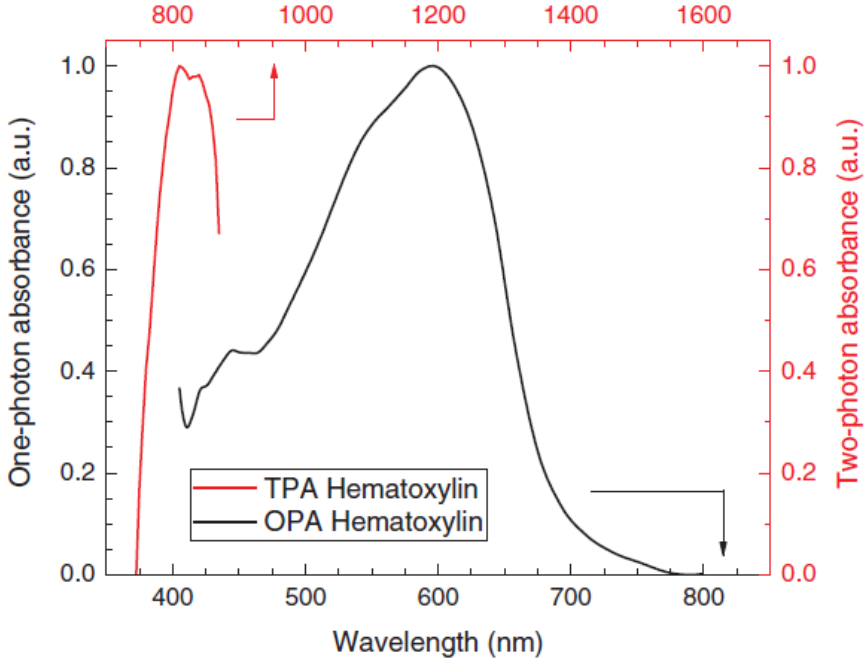
➔ H 2 o.o.m. lower than E

➔ evident concentration quenching
(previosly observed @ lower concentrations)*

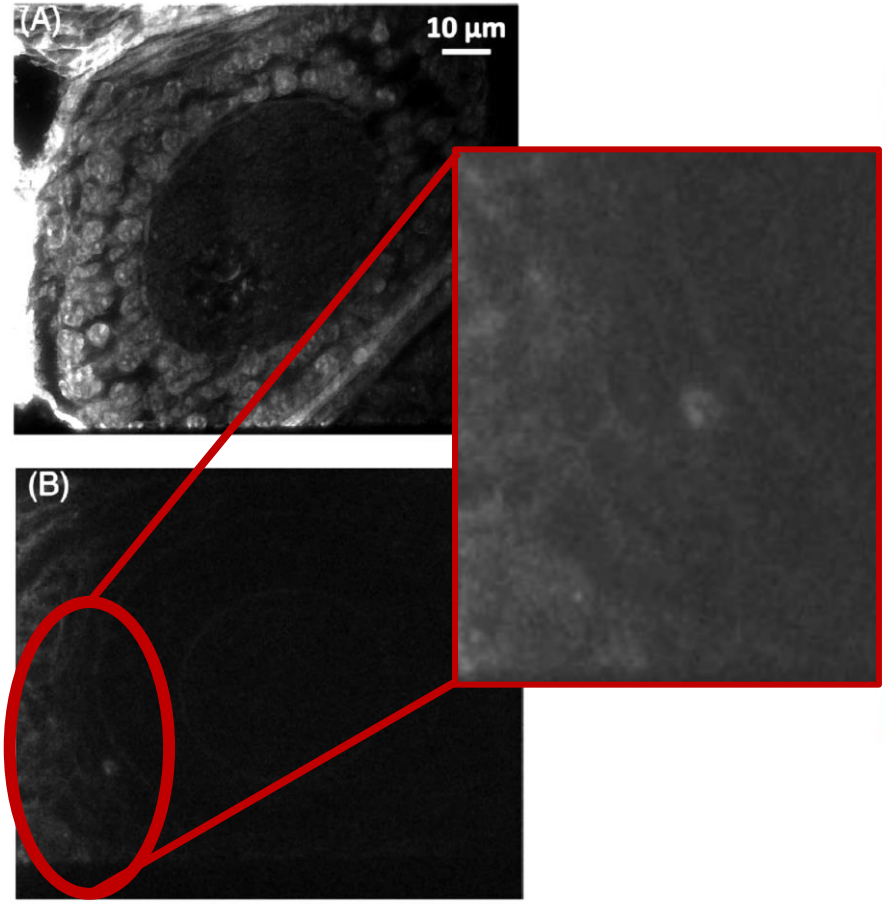
*A. Tuer et al., J. Biomed. Opt. (2010), **15**, 026018

One-photon vs. Two-photon

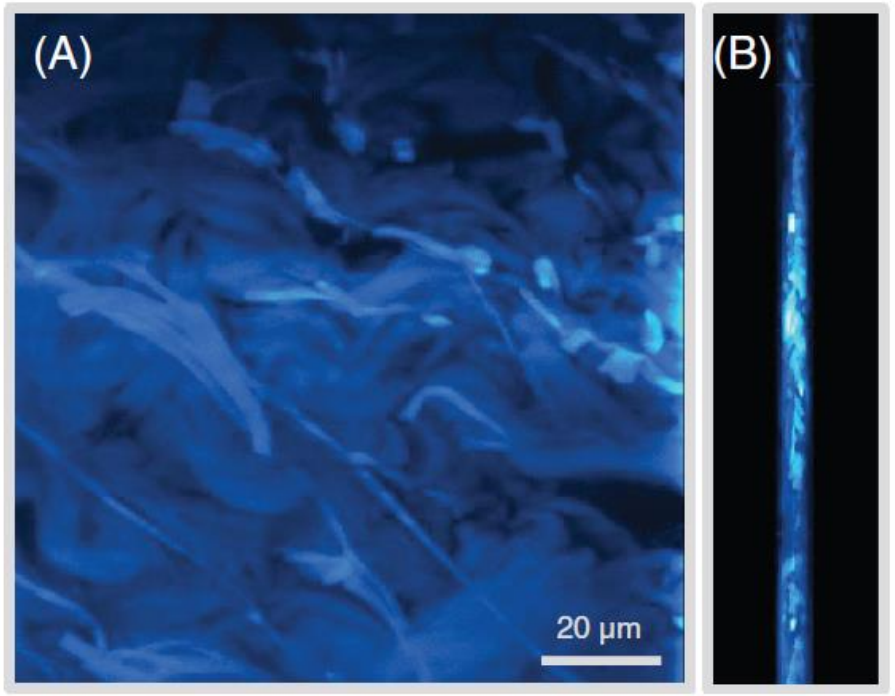




Sample imaging



Mouse ovary cells



Human nevus

Summary

➔ two-photon spectral features od Eosin & Hematoxylin in DI water

➔ confirmed good nonlinear fluorescence in standard λ range

➔ marked concentration quenching for both dyes

➔ E & H staining is a good choice for two-photon fluorescence microscopy

J. Parravicini, et al., Appl. Sci. Vol. 12, 3938 (2022)
J. Parravicini, et al., J. Biophotonics Vol. 13. e202000141 (2020)
E. Hasani, et al., J. Microsc. Vol. 270, 210-216 (2018)

Thanks for your attention

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