Supplementary File 1

**Intravital Deep-Tumor Single-Beam 3-Photon, 4-Photon and Harmonic Microscopy**

Gert-Jan Bakker1, Sarah Weischer1, Júlia Ferrer Ortas2, Judith Heidelin3, Volker Andresen3, Marcus Beutler4, Emmanuel Beaurepaire2 and Peter Friedl1,5,6

1 Department of Cell Biology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Centre, 6525 GA Nijmegen, The Netherlands

2 Laboratory for Optics & Biosciences École Polytechnique, CNRS, INSERM, IP Paris, 91128 Palaiseau Cedex, France

3 LaVision BioTec GmbH, a Miltenyi Biotec company, 33617 Bielefeld, Germany

4 APE Angewandte Physik & Elektronik GmbH, 13053 Berlin, Germany

5 David H. Koch Center for Applied Genitourinary Cancers, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030, USA

6 Cancer Genomics Centre, 3584 CG Utrecht, The Netherlands

**Contact details corresponding authors:** Gert-Jan Bakker: email [gert-jan.bakker@radboudumc.nl](about:blank), P +31 (0)24 36 142 96. Peter Friedl: email [peter.friedl@radboudumc.nl](about:blank), P +31 (0)24 36 109 07. Mail address: Dept. of Cell Biology (283) RIMLS, Radboudumc, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

**Email addresses co-authors:** Sarah Weischer: [sarah.weischer@radboudumc.nl](about:blank), Júlia Ferrer Ortas: [julia.ferrer-ortas@polytechnique.edu](mailto:julia.ferrer-ortas@polytechnique.edu), Judith Heidelin: [heidelin@lavisionbiotec.de](about:blank), Volker Andresen: [andresen@lavisionbiotec.de](about:blank), Marcus Beutler: [marcus\_beutler@ape-berlin.de](about:blank), Emmanuel Beaurepaire: emmanuel.beaurepaire@polytechnique.edu.

**Supplementary File 1a.** Comparison of parameters related to linear, lowIR and highIR excitation modalities.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Modality | Confocal | lowIR (Ti:Sa/OPO) | 1300 nm highIR | 1650 nm highIR |
| Processes | 1-photon, reflection | 2-, 3-photon,  SHG, THG | 3-photon,  SHG, THG | 3-, 4-photon,  SHG, THG |
| Pulse frequency (MHz) | Continuous | 80 | 1 | 1 |
| Pulse length (fs) | - | 140 | 53 | 89 |
| Pulse energy focus (nJ) | - | < 0.4 | < 2 a | < 7 a |
| Peak power focus b | < 1 mW1,2 | < 3 kW | 38 kW | 78 kW |
| Power surface (mW) | < 12 | 40-120 | 2.8-33 | 8.7-38 |
| Emission *I(E) ~ …* | *E* | *E2, E3* | *E2, E3* | *E3, E4* |
| Pixel dwell time (µs) | 2 | 2-4 | 10-20 | 10-20 |
| Attenuation of light | very high | moderate | moderate | low |
| Water abs. sample | ~ 0 % | < 3 % | < 6 % | < 26 % |
| Max. depth in tumor (µm) | < 1002,3 | 255 | 415 | 395 |

a) Derived from maximum tolerable pulse energy (at the tumor surface, focus 50 µm deep in tissue, Figure 2d, h and i) and effective attenuation lengths for tumor tissue (Figure 3e).

b) Derived as pulse energy in the focus divided by pulse duration.

**Supplementary File 1b.** Order of the excitation processes (*n*) from emission intensity as a function of excitation energy (related to Figure 1).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Exc. [nm] | Condition | Hoechst | eGFP | TagRFP | mCherry | AF680 | SHG | THG |
| 1650 | *In vivo* | 4.2±0.2 | -- | 3.2±0.2 | 3.1±0.4 | 3.0±0.3 | 2.15±0.04 | 3.2±0.1 |
| 1650 | Spheroids | -- | 3.8±0.3 | 3.0±0.2 | 3.0±0.1 | -- | -- | 3.0±0.2 |
| 1650 | Spheroidsa) | -- | -- | 3.1±0.1 | 3.0±0.2 | -- | -- | -- |
| 1300 | Spheroids | -- | 2.6±0.4 | 2.5±0.4 |  | -- | 1.85±0.08 | 2.9±0.3 |

a) Measured with the excitation source set to a repetition rate of 0.5 MHz.

**Supplementary File 1c.** Experimental parameters for brain measurements optimized for THG and/or AF680 emission.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Channel / depth / Wavelength [nm] | Line av. | Port | DM | BP | PMT | Imm. | Seq. |
| THG / < 830 µm / 1650 nm | 2 | 2 ch | 525/50 | no | GaAsP | H2O | 1 |
| THG / > 830 µm / 1650 nma) | 6 | 2 ch | 525/50 | no | GaAsP | H2O | 2 |
| THG / > 830 µm / 1650 nm | 6 | 2 ch | 880lp | no | GaAsP | D2O | 3 |
| AF680 / < 830 µm / 1650 nm | 2 | 4 ch | 900lp | 710/75 | GaAs | H2O | 1 |
| AF680 / > 830 µm / 1650 nm | 6 | 2 ch | 880lp | 710/75 | GaAs | H2O | 3 |
| THG / < 500 µm / 1270 nm | 3 | 2 ch | 485lp | 417/60 | GaAsP | H2O | 4 |
| AF680 / < 500 µm / 1270 nm | 3 | 4 ch | 900lp | 710/75 | Alkali | H2O | 4 |
| AF680 / 500-669 µm / 1270 nm | 1 | 4 ch | 900lp | 710/75 | Alkali | H2O | 5 |
| AF680 / > 669 µm / 1270 nm | 3 | 4 ch | 900lp | 710/75 | Alkali | H2O | 6 |

a) Solely used for generation of the 1650 nm signal attenuation curve.

**Additional References**

1. Pawley, J. B. *Handbook Of Biological Confocal Microscopy*. (Springer, 2006).

2. Centonze, V. E. & White, J. G. Multiphoton excitation provides optical sections from deeper within scattering specimens than confocal imaging. *Biophys. J.* **75**, 2015–2024 (1998).

3. Helmchen, F. & Denk, W. Deep tissue two-photon microscopy. *Nat. Methods* **2**, 932–940 (2005).