Supplemental Document

Biomedical Optics EXPRESS

Fast *in vivo* multiphoton light-sheet microscopy with optimal pulse frequency: supplement

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Supplement DOI: https://doi.org/10.6084/m9.figshare.13003595

Parent Article DOI: https://doi.org/10.1364/BOE.400113

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Supplementary figures

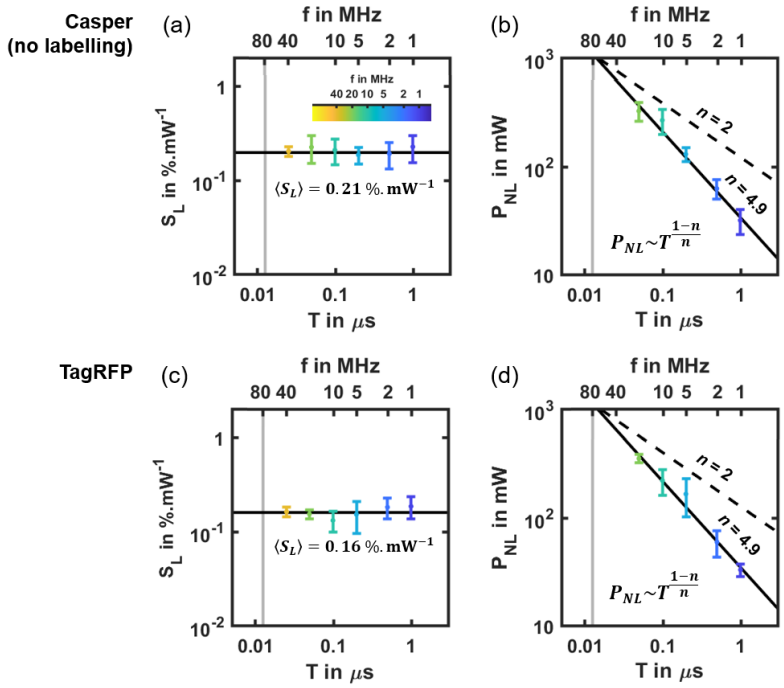


Fig. S1. Linear effect and nonlinear photodamage threshold in unlabeled and TagRFP labeled embryos. (a) Linear slope S_L of the HBR relative variation of unlabeled *casper* zebrafish hearts (N = 32 embryos) depending on the laser pulse frequency $f = 1/T$. Black line indicates mean value $\langle S_L \rangle = 0.211 \pm 0.017\ \%.\mathrm{mW}^{-1}$). (b) Nonlinear photodamage threshold P_{NL} in unlabeled hearts (N= 27 embryos) depending on the laser pulse frequency $f=1/T$. Black line shows the result of the scaling law fitted on logarithmic scaled data. $P_{NL}(T)$ follows a scaling law of order n~4.9. (c) Linear slope S_L of the HBR relative variation of TagRFP labeled zebrafish hearts (N = 27 embryos) depending on the laser pulse frequency $f = 1/T$. Black line indicates mean value $\langle S_L \rangle = 0.161 \pm 0.014\ \%.\mathrm{mW}^{-1}$). (d) Nonlinear photodamage threshold P_{NL} in TagRFP labeled hearts (N = 23 embryos) depending on the laser pulse frequency $f = 1/T$. Black line shows the result of the scaling law fitted on logarithmic scaled data. $P_{NL}(T)$ follows a scaling law of order n~4.9. Error bars indicate standard deviation. Black dashed line indicates a scaling low of order $n = 2$ to show how it deviates from 2PEF signal. Results of scaling law fits are listed in Table S2.

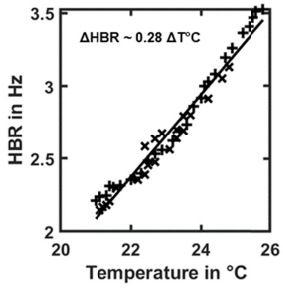


Fig. S2. Heart beat rate depending on temperature for 4-5 days post-fertilization zebrafish embryos. The embryos were placed in warm water and their HBR was measured as the water cooled down. HBR increases linearly with temperature. Black line indicates the result of a linear fit (ΔHBR in Hz = 0.285 ± 0.015 ΔTemperature in °C, R^2 = 0.97).

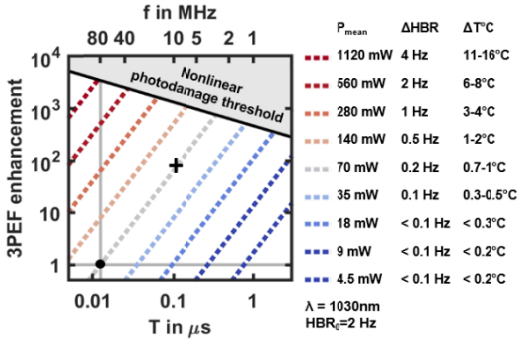


Fig. S3. 3PEF signal enhancement graph used to select the optimal laser pulse frequency. Signal enhancement corresponds to $\frac{T^2 P_{mean}{}^3}{T_0{}^2 P_0{}^3}$ with $T_0 = 1/f_0 = 1/80$ MHz and $P_0 = 70$ mW (black dot). Solid black lines correspond to the nonlinear photodamage threshold. Dashed blue-to-red lines corresponds to line of constant mean power of slope 2 and constant linear effect (variation of heart beat rate ΔHBR and of temperature ΔT°C). Black cross, imaging conditions used in Fig. 6b.

Supplementary tables

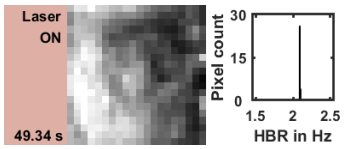
Figure	Experiment	f	P_{mean}	Laser scan speed	Field of view	Frame rate
		MHz	mW	µm.ms^{-1}	pixel^{2}	frame.s^{-1}
1a	$2PEF$ signal	4.4 *to* 40	100	40	500×500	168
1b	SHG signal	0.6 *to* 40	100	8	2048×2048	41
1c	$3PEF$ signal	4 *to* 13	54	40	500×500	33
4a	Nonlinear photodamage threshold	1 2 5 10 20	29 51 126 201 322	40	500×500	168
S1b		1 2 5 10 20	32 63 131 269 327	40	500×500	168
S1d		1 2 5 10 20	33 60 166 221 353	40	500×500	168
5a	Photobleaching experiment	0.6 2 5 10 20 40	15 28 45 63 90 127	40	500×500	168
6b Vis. 3	4D heart *in vivo* imaging	10	70	40	500×500	168
Vis. 4		10 or 40			500×500	155
Vis. 5		10			400×148	488

Table S1. Experimental parameters. f and P_{mean} are the laser repetition rate (or pulse frequency) and mean power, respectively. Vis. For visualization.

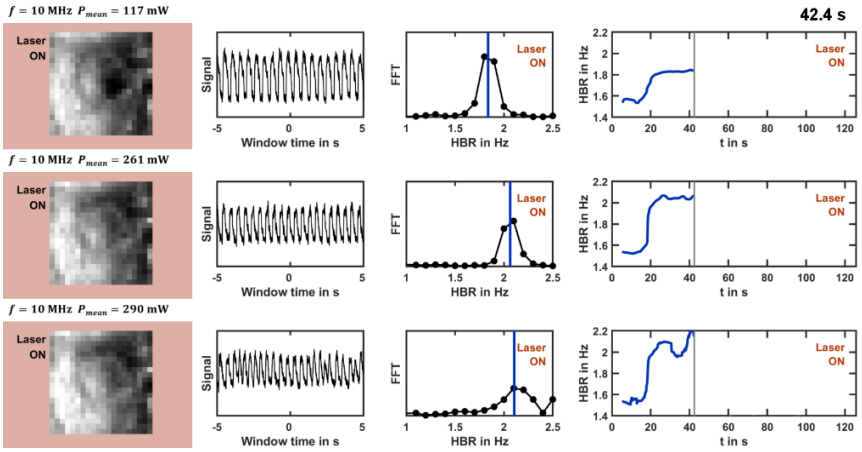
Fig.	Optical effect	Scaling law	P_{mean}	Sample	A	B	R^2	n	n: 90% conf. interval
		$Optical\ effect \sim T^{?}$			Linear regression: $log(Optical\ effect) = A\ log(T) + B$				
1a	$2PEF$ signal	$2PEF \sim T^{n-1}$ $n = A + 1$	$P_{mean} = cst$	mCherry embryos	1.2	2.1	0.91	2.2	[1.9, 2.5]
1b	SHG signal	$SHG \sim T^{n-1}$ $n = A + 1$	$P_{mean} = cst$	KTP nanocrystals	1.0	1.9	0.998	2.0	[2.0, 2.1]
1c	$3PEF$ signal	$3PEF \sim T^{n-1}$ $n = A + 1$	$P_{mean} = cst$	Fluo-spheres	2.0	2.0	0.999	3.0	[3.0, 3.1]
3a	Linear effect S_L	$S_L \sim T^{n-1}$ $n = A + 1$	P_{mean} varies to estimate S_L	mCherry embryos	0.032	1.3	0.016	1.0	[0.95,1.1]
S1a				Unlabeled embryos	−0.009	1.3	0.002	0.99	[0.92,1.1]
S1c				TagRFP embryos	0.047	1.2	0.05	1.0	[0.98,1.1]
4a	Nonlinear photo-damage threshold P_{NL}	$P_{NL} \sim T^{\frac{1-n}{n}}$ $n = 1/(A + 1)$	$P_{mean} = P_{NL}$	mCherry embryos	−0.83	1.5	0.98	5.8	[4.4, 8.2]
S1b				Unlabeled embryos	−0.80	1.5	0.93	4.9	[3.6, 7.8]
S1d				TagRFP embryos	−0.80	1.5	0.90	4.9	[3.3, 9.8]
5b	Photo-bleaching rate k	$k \sim T^{\frac{n}{2}-1}$ $n = 2A + 2$	$P_{mean} \sim T^{-2}$	mCherry embryos	0.67	−2.46	0.99	3.3	[3.2, 3.5]

Table S2. Scaling laws of optical effects and estimation of their *n*-order using linear regression of logarithmic scaled data. *T* and P_{mean} are the laser pulse period and mean power, respectively.

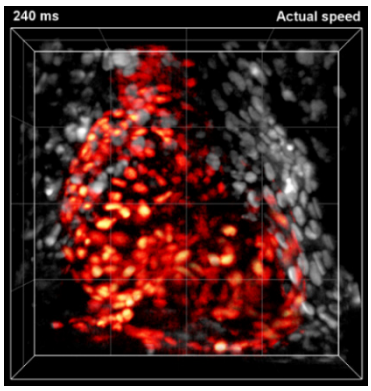
Supplementary visualizations



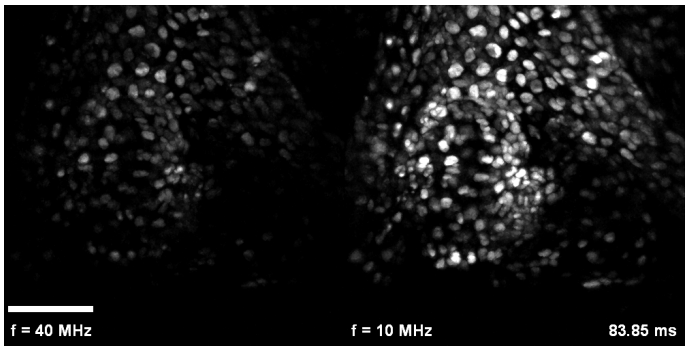
Visualization 1. Estimation of instantaneous HBR. A sequence of white light illumination images of the embryonic heart (left) is used to estimate the instantaneous HBR. HBR histogram from the 30 best pixels (right) demonstrate the good precision of the measure.



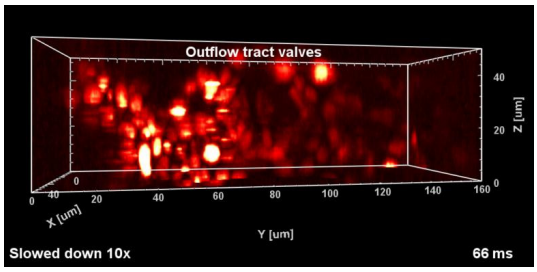
Visualization 2. Experimental workflow of HBR analysis. The analyses of three typical acquisitions, at P_{mean} = 117 mW (top), 261 mW (middle) and 290 mW (bottom) on the same zebrafish heart at f = 10 MHz are presented. First column: white light illumination images of the heart of the embryo. Second column: periodic signal fluctuation extracted from individual pixels over a 10 s window. Third column: windowed Fourier transform of the signal to extract of the HBR over that window. Bottom line: HBR as a function of time. Nonlinear photodamage are observed at P_{mean} = 290 mW with heart beat arrhythmia followed by intense signals.



Visualization 3. 4D reconstruction of the zebrafish beating heart imaged with 2P-SPIM at 168 frames per second with optimized laser parameters. Histone mCherry-labeled zebrafish embryo at 3 days post-fertilization imaged using f = 10 MHz and P_{mean} = 70 mW with 200x200x100 µm or 500x500x100 voxels field-of-view. Heart cells in red were manually segmented. Grid spacing of 50 µm.



Visualization 4. 2PEF signal enhancement using f = 10 compared to f = 40 MHz laser pulse frequency at constant mean power. 4D reconstructions of the zebrafish beating heart imaged with 2P-SPIM at 155 frames per second (74 frames per cardiac cycle). Histone mCherry-labeled zebrafish embryo at 4 days post-fertilization imaged using f = 40 MHz (left) or f = 10 MHz (right) and P_{mean} = 70 mW with 200x200x100 µm or 500x500x100 voxels field-of-view. Cardiac cycles were manually synchronized. Movie speed slowed down 6.2 times compared to actual speed. Scale bar of 50 µm.



Visualization 5. 4D reconstruction of the outflow tract valves in the zebrafish beating heart imaged with 2P-SPIM at 488 frames per second with optimized laser parameters. Histone mCherry-labeled zebrafish embryo at 4 days post-fertilization imaged using f = 10 MHz and P_{mean} = 70 mW with 160x59x50 µm or 400x148x50 voxels field-of-view.