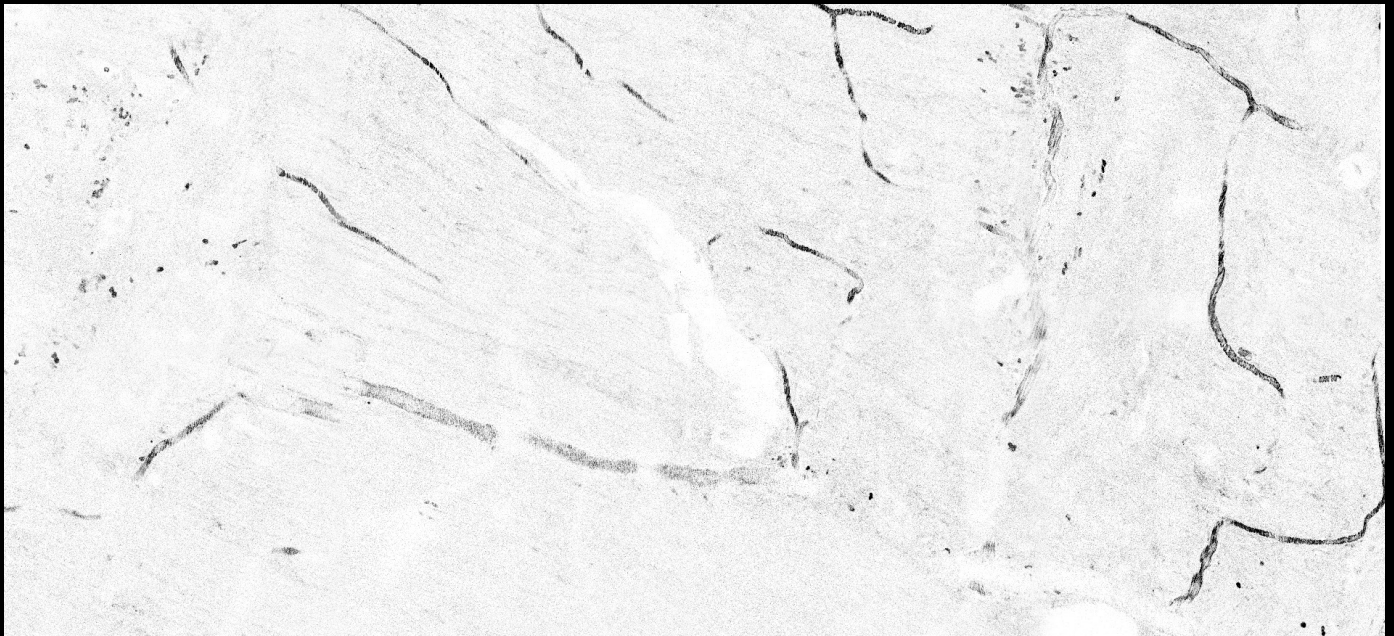


**Application Note**

# SP8 DIVE: LABEL-FREE BLOOD FLOW QUANTIFICATION

Label Free Blood Flow Quantification

Third Harmonic Generation (THG) Imaging in Biology



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## SP8 DIVE: Label-free microscopic blood flow quantification using Third Harmonic Generation (THG) imaging

### How 4Tune (spectrally tunable non-descanned detection) and long IR excitation simplify blood flow quantification in vivo

This application note is a guide for data acquisition and analysis of label-free blood flow tracking using THG. This is enabled by long IR excitation wavelengths and easy-to-use spectral non-descanned detection. Exploiting the intrinsic contrast of red blood cells, labeling becomes redundant which does not only save time but also the reduces the risks of artifacts induced by the label or the labeling procedure.

Viability of mammalian tissues is categorically dependent on blood flow. The rate of blood flow can therefore serve as a valuable measure to determine the overall status of an organism, organ or tissue with implications in mouse models in various kinds of biological research: Neuroscience, immunology, cancer research. An increase or decrease of the blood-flow rate can point towards cardiac diseases, poor state of vessels prone to stroke, formation of blood clots or other cardiac conditions.

In most cases, fluorescent labels like microscopic beads, labelled large molecules or labelled erythrocytes are injected into the blood system and used for the analysis of the blood flow. These methods have proven to be reliable, reproducible and superior to earlier approaches using radioactive tracing.<sup>[1]</sup> However, they require the injection of labels into the blood stream with the potential to alter physiology. Further, the labels can only be imaged for a limited time frame, until they are metabolised by the organism. This is a major problem if blood flow is supposed to be used as a measure of vitality of the specimen in longer imaging sessions of multiple hours. In addition, the use of a fluorescent channel for blood flow determination limits the use of additional markers in the same experiment, especially when imaging intravascular targets. Furthermore, their preparation uses additional resources.

The advantage of a label-free method for blood flow tracking and analysis is obvious. Luckily, a contrast agent is intrinsically present in blood stream: red blood cells. The refractive index mismatch between red blood cells and their surroundings can be visualized using *Third Harmonic Generation* contrast (THG).<sup>[2]</sup> THG occurs at refractive index mismatches and is observed at a third of the pulsed excitation light wavelength. In a common multiphoton microscope equipped with a suitable laser, this can be realized using an excitation wavelength between 1200 and 1300 nm (e.g. OPO based laser or corresponding one-box-lasers) and detected at 1/3 of the excitation wavelength. For example, using multiphoton excitation at 1200 nm and a detection band around 400 nm, which is achieved most easily by using tunable detectors creating a narrow detection window around 400 nm.

### At a glance: THG in biological imaging<sup>[3]</sup>

- > THG stands for Third Harmonic Generation
- > Occurs at refractive index mismatches, e.g. a water-lipid interface, interface between red blood cells and surroundings
- > Non-fluorescent process
- > Can be observed at 1/3 of the exciting wavelength
- > Occurs mostly in forward direction but backscattered signal can be collected in epi direction, especially from thick samples<sup>[4]</sup>
- > Hardware suggested:
  - A fs-pulsed laser capable of emitting between 1200 and 1300 nm
  - A sensitive detector capable of detecting around 400 nm, tunable or with special filter

## Data acquisition

### General imaging parameters

To analyze the blood flow rate, only a few parameters need to be measured: The diameter of the blood vessel/capillary and the speed of the blood cells flowing through it.

To visualize the blood vessels label-free, the following parameters are suggested:

- > Objective lens: HC FLUOTAR L 25x/0.95 WATER or similar
- > Excitation wavelength: 1275 nm
- > Detection range 4Tune spectral NDD: 420-430 nm
- > Resolution: 512 px x 512 px
- > Speed: 8 kHz resonant bidirectional; averaging as needed, e.g. 16 x line averaging to collect images, 4 x averaging for time series and xt scans.

The overview was acquired using the parameters above and 16x line averaging, it shows capillaries in the large intestines of a mouse. Image size was 1400 x 765  $\mu\text{m}^2$ , acquired with LAS X Navigator functionality.

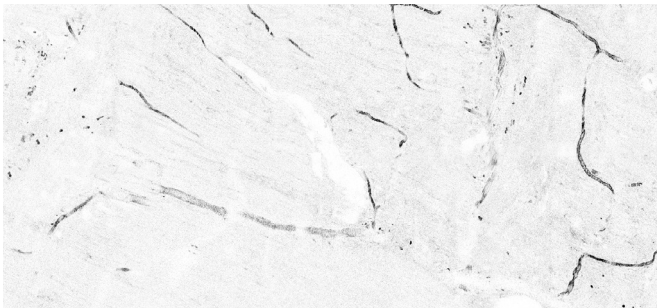


Figure 1: 1400 x 765  $\mu\text{m}^2$  tile scan of large intestine of an anesthetized mouse. The blood system (black) is visualized using the intrinsic label-free THG signal. Excitation was 1275 nm, non-descanned detection 420-430 nm.

### How to acquire time resolved data to analyse the velocity of the red blood cells

The velocity of the blood cells can be measured directly using the xt-scan modality. The vessel should be aligned horizontally along the x-axis using the Abbe-Königs-rotator of the SP8 DIVE. With switching from the xy- to the xt-scan mode, a line appears which indicates the position of the xt-section. This section should be aligned to the center of the vessel:

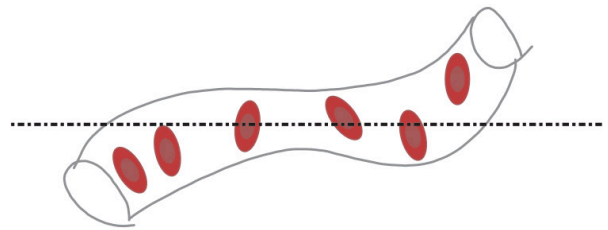
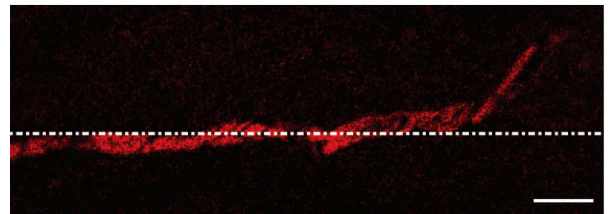


Fig. 2: When switching to the xt-scan modality, a line appears that indicates the x-section of the scan. The line should be aligned to the vessel of interest.

In the xt scan, the intensity along the defined section will be recorded over time. Since blood flow is a fast event, the recording of seconds is typically sufficient to enable proper analysis. Ideally, the recording time is long enough to follow one blood cell over the whole length of the x-section.

### Acquisition of a smooth image to determine the diameter of the blood vessel

To determine the diameter of the vessel a low-noise image is required. This should ideally be a z-stack. However, in dynamic tissue such as large intestines, the movement of the specimen can make this impossible. In this case, a two-dimensional image should be used and the diameter should be averaged over multiple positions and timepoints to prevent artifacts from moving in and out of the z-plane.

### At a glance: 4Tune – spectrally tunable NDD

- > Equipped with variable dichroic splitters and variable bandpass filter
- > Controlled by the LA S X software
- > Can be adjusted to the detection window needed for the application
- > Up to 4 channels can be configured, each one can be equipped with either a HyD for most sensitive detection or a PMT for the largest dynamic range.
- > Narrow windows down to 10 nm width – optimal for collection of THG signals – can be accomplished with a few mouse clicks and without the need of a special hardware filter
- > Stepsizes as precise as 1 nm are possible
- > Emission lambda scans in non non-descanned mode are easily done

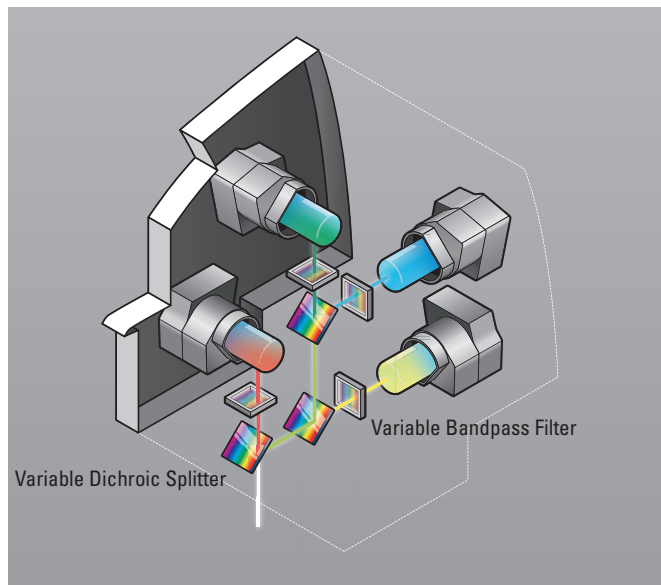


Figure 3: Scheme of the spectrally tunable 4Tune detector: White emission light is split onto up to 4 channels depending on the setting of the variable dichroics and bandpass filter.

## Data analysis

### Blood flow velocity

Blood flow velocity ( $V$ ) is defined as distance travelled per time and often expressed as cm/s in larger vessels or mm/s in capillaries.

$$V = \frac{\text{distance}}{\text{time}}$$

The velocity is calculated from the xt-kymograph. The scalebar drawn along the line created by the moving blood cell gives it directly, however in general, averaging over multiple positions is advised.

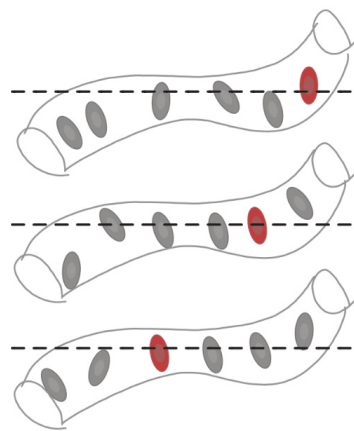
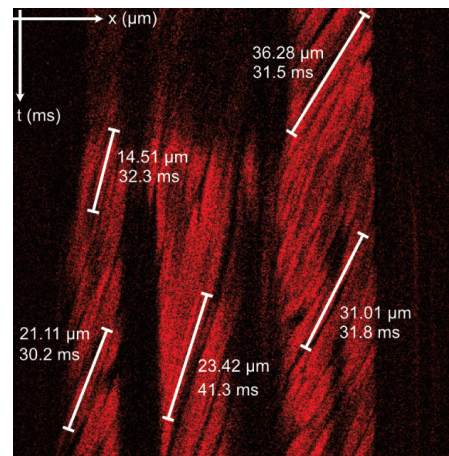


Fig. 4: The blood cells travelled 0.8 mm/s on average.

Because all capillaries are of similar size, blood flow velocity is a meaningful parameter often reported in the literature.<sup>[5]</sup> For larger vessels however, the total amount of blood that flows through a vessel is not only dependent on flow velocity but also the size of the vessel the blood is flowing through at said velocity. Therefore, the blood flow rate (Q), which is the product of blood flow velocity (V) and the blood vessel cross-sectional area (A) is of more interest. Blood flow rate can be calculated from the same dataset when in addition to velocity the diameter or radius (r) of the vessel of interest is measured.

$$Q = V * A$$

By substituting with the formula from above and the area of a circle formula we get

$$Q = \frac{\text{distance}}{\text{time}} * \pi * r^2$$

**Vessel diameter (radius)**

It is crucial to measure the diameter of the vessel correctly. In a dynamic specimen, this is best accomplished by averaging over either multiple positions and/or multiple time points.

Using the scalebar in LAS X, the diameter can easily be determined at e.g. 10 positions. For this dataset, the diameter at 10 different positions were measured and the average vessel diameter was calculated to 6.52 μm.

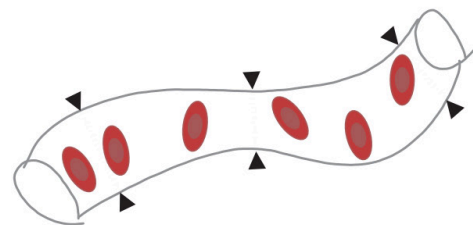
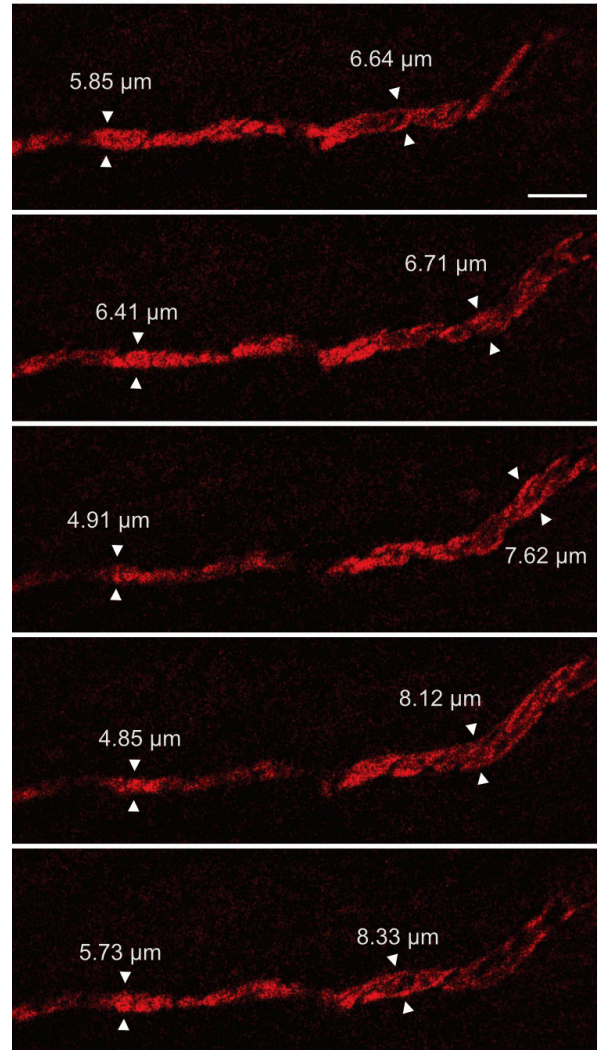


Fig. 5: The diameter of the vessel was measured at different positions and time points to determine the average diameter.

### Blood flow rate

Using the above formula, the blood flow rate is calculated. It is typically reported in mL/min:

$$\begin{aligned}
 \text{blood flow rate } \left[ \frac{\text{mL}}{\text{min}} \right] &= \frac{\text{distance travelled } [\mu\text{m}] \cdot \pi \cdot (0.5 \cdot \text{vessel diameter } [\mu\text{m}])^2}{\text{travel time } [\text{ms}]} \cdot 10^{-12} \left[ \frac{\text{mL}}{\mu\text{m}^3} \right] 6 \cdot 10^4 \left[ \frac{\text{ms}}{\text{min}} \right] \\
 &= 1.14 \cdot 10^{-6} \left[ \frac{\text{mL}}{\text{min}} \right]
 \end{aligned}$$

The diagram includes the following annotations:

- Averaged value from data in  $\mu\text{m}$  (pointing to distance travelled)
- Averaged value from data in  $\mu\text{m}$  (pointing to vessel diameter)
- Averaged value from data in ms (pointing to travel time)
- Factor to translate  $\mu\text{m}^3$  to mL (pointing to the  $10^{-12}$  conversion factor)
- Factor to translate ms to min (pointing to the  $6 \cdot 10^4$  conversion factor)

This value is in agreement with the value obtained by an experiment with labelled erythrocytes.

## Discussion and Summary

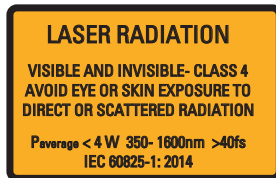
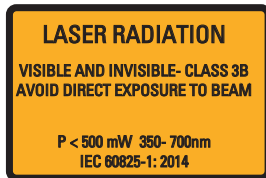
This application note is a guide through the steps needed to acquire and analyse blood flow velocity and blood flow rate. Measurement is done exploiting intrinsic label-free contrast from the red blood cells and can be done without invasive labeling. This approach is advantageous when combined with in vivo microscopy because it can be added to any combination of fluorochromes. Care needs to be taken with longterm blood flow analysis as longterm imaging using THG might lead to photodamage due to high laser intensities needed for a third order excitation process, which in turn may affect local hemodynamics.

Penetration depth of THG microscopy is, as any kind of light microscopy, highly dependent on the nature of the tissue. In a third order excitation process two competing effects have influence on the depth penetration: First, the long IR excitation light easily penetrates deep into scattering tissue. Second, the resulting THG signal light is blue shifted compared to most fluorescent signals and therefore doesn't penetrate (out) as well as the exciting light. Care needs to be taken to choose the appropriate objective lens with color correction (especially in transmission) over this range of wavelengths, e.g. Fluotar VISIR class objectives. Optimizing all conditions, penetration down to several hundreds of micrometers is possible and has been shown in young mouse brain tissue.

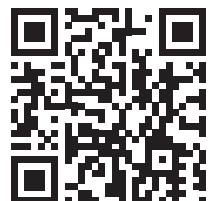
With a state-of-the-art MP microscope equipped with a long-IR laser and flexible detection, the implementation of the workflow presented is straight forward and doesn't require any additional hardware or software.

## References

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- [4] Signal epidetection in third-harmonic generation microscopy of turbid media, D. Débarre et al, Opt. Express 15, 8913-8924 (2007).
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