Light sheet Raman micro-spectroscopy: supplementary material

WALTER MÜLLER,^{1,2} MARTIN KIELHORN,² MICHAEL SCHMITT,¹ JÜRGEN POPP,^{1,2}
AND RAINER HEINTZMANN^{1,2,*}

¹ Institute of Physical Chemistry and Abbe Center of Photonics, Friedrich Schiller University Jena, Helmholtzweg 4, 07743 Jena, Germany

² Leibniz Institute of Photonic Technology, Albert-Einstein Str. 9, 07745 Jena, Germany

*Corresponding author: rainer.heintzmann@uni-jena.de

Published 18 April 2016

This document provides supplementary information to “Light sheet Raman micro-spectroscopy,” <http://dx.doi.org/10.1364/optica.3.000452>. Here we discuss technical details of our setup, geometrical properties of the imaging spectrometer and important steps of data processing. © 2016 Optical Society of America

<http://dx.doi.org/10.1364/optica.3.000452.s001>

Light sheet generation

Our LSRM (Fig. 1d) illuminates the sample with a 577 nm Laser (Coherent Genesis CX 577-2000 STM) of up to 2 W output power. The light sheet is generated by a cylindrical lens and projected by a low NA water immersion objective (Nikon CFI Plan Fluor W 10× N.A. 0.30 WD 3.50 mm) into the sample [1]. Under ideal circumstances, the FWHM thickness of the light sheet in the middle of the field-of-view is 3.1 μm and 10.5 μm on the edge, whereas the FWHM length is 250 μm. We estimate the maximum irradiance in the sample to be 1.7 mW/μm² using full 2 W of output laser power. The whole illumination optics is mounted in an optical cage system.

The sample is observed orthogonally to the illumination light sheet with a high NA water immersion objective (Nikon CFI Apochromat 40× W NIR N.A. 0.80 WD 3.50 mm). The size of the field-of-view, limited by the camera, is 325 μm × 325 μm. Before the Stokes scattered light is spectrally analysed, a long-pass filter of optical density >6 (Semrock ET 590 LP) at 577 nm with a cut-off of 593 nm (rel. 460 cm⁻¹) removes the Rayleigh scattered light.

Fourier-transform imaging spectrometer

The interferometer [2] consists of two silver coated, reduced corner cube reflectors and two 50% beam splitters, placed in the infinity corrected beam path between a high NA objective and its corresponding tube lens. This simple geometry has the advantage of robustness and short optical paths, which leads to a large optical throughput (number of mathematical independent spectra).

The features of this type of interferometer are:

- Inherent optical stability against pitch and yaw of the reflectors

- Stability against transversal shift and roll of reflectors because its placement in the infinity beam path
- Short optical paths between the beam splitters allow steep incident angle and thus large field-of-view
- No adjustment required in case of unified beam splitters
- No loss of contrast by polarisation mixing in the corner-cube reflections
- Two input paths and two output paths (like a Mach-Zehnder interferometer)

The longitudinal precision of the reflector position and thus the optical path difference (OPD) is supervised by a 543 nm reference helium-neon laser (Lasos LGK 7770). All optical components of the interferometer are made from solid blocks of BK7 glass, which leads to reduced angles of divergence in comparison to air.

The maximum isotropic angle of acceptance of the interferometer is limited by the depth of the corner cube reflector and the used clear aperture of it to:

$$\alpha = \pm \tan^{-1} \frac{\sqrt{2}}{6} \approx 13.26^\circ \quad (\text{S1})$$

in the media of the reflector with refractive index $n = 1.516$ and in front of the reflector to:

$$\beta = \sin^{-1} \left(\frac{n}{\sqrt{19}} \right) \approx 20.35^\circ \quad (\text{S2})$$

For high optical throughput, this angle could be utilized most efficiently in a symmetrical 4f setup, while the stop lies in the reflector (Fig S1).

Fig. S1: Unfolded interferometer in a schematic 4f setup (blue arrows): left and right grey squares are beam splitter cubes with refractive index n and size d , centre grey rectangle is the unfolded corner-cube reflector with refractive index n and diameter a of full aperture, red indicates light under highest possible angle of acceptance (α with refractive index n , β in air), A indicates the aperture. The advantage of using reduced corner-cube reflectors is the minimization of distance b between beam splitter cube and reflector (compare also Fig. 1c, 1e).

Interferogram acquisition

The interference patterns of the Fourier-transform imaging spectrometer are observed by an sCMOS camera (Hamamatsu ORCA-Flash 4.0 V2), currently in only one of the output arms. We run the interferometer in step scan mode to guarantee delta-comp-like sampling. Every image of an interference pattern corresponds to a precisely, dynamically adjusted (OPD) as determined by a 543 nm Helium-Neon reference laser (see Active OPD stabilization). This delta-comb like sampling avoids sampling-induced low-pass filtering, which would be detrimental to an aliased under-sampling of the strongly band-limited Raman signal. With our choice of laser wavelengths, we halve the amount of required images by only acquiring images at every second zero crossing of the reference laser (Fig. 1f). The spectrum covers a wavelength range from 1086 nm to 543 nm (i.e. relative wavenumbers from -1085 cm^{-1} to 8123 cm^{-1} with reference to 577 nm). One advantage of the FT approach in contrast to grating based spectrometers is the equidistant wavenumber scale. Thus, the knowledge of the reference laser wavelength is sufficient for calibration. Due to noise advantages, we record fully double-sided interferograms [3]. Typical interference contrast achievable with our setup is 92 %.

Active OPD stabilization

As a drive for one of the reduced corner-cube reflectors we use a piezo stick-slip motor stage (PI miCos LPS-45). We have realised a feed-back-loop to stabilise the OPD during image acquisition by measuring the interference intensity of the reference laser on both interferometer outputs with two BPW 34 photodiodes, two TL072 OPVs and the on-chip analogue to digital converter of an Arduino Mega (Microcontroller: ATmega1280 8-Bit Controller). The information is processed in real-time on the Arduino with 32 bit float precision and fed back to the motor stage via RS232 commands. This technique allows keeping the OPD broadband stable (100 Hz) to a precision better than 5 nm on every zero crossing. As a result, we are able to sample the interference with a comb function, having peaks smaller than 5 nm in with an absolute accuracy, which is limited by the helium-neon laser stability.

A drawback of the used drive is its unpredictable stick-slip behaviour and a $20 \text{ }\mu\text{m}$ sinusoidal absolute position-mismatch modulation due to the inner position encoder, which limits speed of operation and the length of under-sampling jumps.

Sample preparation

As common in light sheet illumination microscopy [4] we suspend the sample in a water-filled chamber. For thermo-mechanical stability we embed the sample in 2 % standard agarose to hold it in a spoon-like aluminium structure (Fig. 1c)

instead of using a glass capillary equipped with a plunger. With this way of sample preparation, we reduce the drift of a, transparent biological sample e.g. an unlabelled paraformaldehyde fixed zebrafish in water immersion with our highest achievable irradiance of $1.7 \text{ mW}/\mu\text{m}^2$ to less than $1 \text{ }\mu\text{m}$ per hour with $23 \text{ }^\circ\text{C}$ ambient temperature.

Data processing of interferograms

As a first step we normalize the acquired interference image OPD-stack. We subtract an averaged dark image and divide by a flat-field image. The flat-field image of the system was acquired as the background subtracted mean of 1000 images of a clear water sample (removing the lowest and highest 10 % quantile in each pixel) at a constant large OPD. This flat-fielding effectively normalizes the sCMOS camera at its approximated later working intensity, compensating for pixel sensitivity and gain differences introduced by dust near the image plane and inhomogeneous detector quantum efficiency. It also corrects for the irradiance decay of the light sheet in water caused by the Gaussian profile.

The normalisation is followed by a lateral sample-drift correction. Operating our system under the described conditions typically leads to a few pixels ($< 1 \text{ }\mu\text{m}$) lateral drift over 30 min. We detect this drift over averages of 8 consecutive images by cross-correlating to the first average of 8 images using “findshift” (DIPimage Matlab toolbox; <http://www.diplib.org/dipimage>) and correct for the drift using the Fourier-space based “shift” routine.

By estimating and subtracting the exponential decay over time of residual fluorescence, a pixel wise correction of slowly fading background is performed in every interferogram. This reduces an artefact pseudo-signal occurring around the zero frequency in the results. In case of under sampling at half the Nyquist frequency, these bleaching effects occur near the reference wavelength (Fig. 1f). In our case the pseudo-signal stays sufficiently apart from the Raman spectral information.

After these corrections the spectral information is obtained by a pixel wise Fourier-transformation. To avoid Fourier series artefacts, a window function is applied were $1/16^{\text{th}}$ of the most negative and positive OPDs are blended (Hanning window) to their average. The result is a hyperspectral image, containing a complex valued spectrum in each pixel from -1085 cm^{-1} to 8123 cm^{-1} with reference to 577 nm.

Data processing of spectra

Each of the spectra should have a real part only. However, due to the OPD not being precisely centred, the information is in praxis spread into real and imaginary part by a phase, which is non zero [3]. At first glance it would be sufficient to ignore the phase information and to only consider the absolute value of each frequency. However, in the presence of noise, taking only the real part of prior phase-corrected spectra lead to much better performance especially were the signal is low. Two mathematically identical approaches to correct for a wavelength dependent phase are known [5,6] and compared [7], working either in real- or in frequency space. In general the instrument-phase-function of an imaging system is not only wavelength dependent but also slowly varying in space. For our data, which contains thousands of spectra, we implemented an approach in frequency space by only taking advantage of the slowly varying phase in space and wavelength. For this, we use the Fourier-transformed hyperspectral complex image and divide it by a 2+1D-Gaussian-blurred and normalized version of itself (typ. standard deviation of the Gauss $\sigma_x \cdot \sigma_y \cdot \sigma_\lambda = 20 \cdot 20 \cdot 4 \text{ voxel}$). This averages the phase noise over many voxels and orients the

real axes for every single voxel along the direction of the actual signal. This method proved to be very robust in our hands and has the advantage, that every dataset is self-phase-correcting without the need for calibration experiments. In the worst case, if the signal of all blurred neighbour pixels is more than:

$$\sqrt{2\sqrt{\pi}\sigma_x\sigma_y\sigma_\lambda} = 75 \quad (\text{S3})$$

times below the noise of a single pixel, the real axis could be turned arbitrarily into wrong directions, but this happens only in situations, where the signal is practically useless anyway. By choosing the real part of the phase-corrected data (instead of the absolute value), we eliminate the positive contribution of the RMS noise and end up with a constant Gaussian noise centred around zero. It can again average out by binning, as it is essentially bias-free.

In our imaging setup we use our interferometer at a range of input angles α of ± 2.6 degrees (diagonal of field-of-view). This leads to a position-dependent sampling frequency of the interference pattern scaling with $1/\cos(\alpha)$ [3]. To avoid a position-depending wavelength scale, we correct for this input-angle effect in case of high resolution (here $< 20 \text{ cm}^{-1}$) and large field-of-view (here $> 300 \mu\text{m}$) measurements. The parameters for this correction can be gained by pure geometry without calibration. Only in case of an optical axis mismatch between objective lens and tube lens it is necessary to determine once the $\alpha = 0$ position on the camera chip. We perform the correction by a stretching of the spectrograms with a Lanczos 8 core interpolation.

After this raw data processing, various spectral analysis methods can be applied.

Data visualisation

We are facing hyperspectral images containing up to four million pixel and 2048 different spectral canals for each slice of the sample. Not all the information is independent and it is overlaid by noise. Feeding a complete 50 slices containing stack of hyperspectral images at 18 cm^{-1} into an NMF algorithm is not computationally feasible with our PCs (19 GB for fingerprint and CH stretch modes only, in single precision). We instead used a single, boarder-cropped slice of zebrafish data recorded at 4.4 cm^{-1} spectral resolution to be separated into five components by NMF. These were spectrally binned later fourfold to match the spectral resolution (18 cm^{-1}) of the stack. The abundances were calculated by applying the pseudoinverse of the so-obtained mixing matrix to the entire stack.

After the separation, we applied a 3D *a posteriori* maximum likelihood deconvolution [8] with the ideal PSF calculated for the centre of the light sheet. During the deconvolution process, we apply a positivity constraint and a modified Good's roughness [9] regularisation P_{mGR} to each channel separately:

$$P_{mGR}(f) = \lambda_{mGR} \sum \frac{|\vec{\nabla} f|^2}{|f| + \epsilon_{mGR}} \quad (\text{S4})$$

where f is the object estimate, λ_{mGR} the regularisation weight and ϵ_{mGR} a factor [10] that ensures differentiability and takes care for Gaussian sensor noise. We used fixed values of $\lambda_{mGR} = 0.008$ and $\epsilon_{mGR} = 0.1$.

We think that applying NMF before deconvolution is suitable, because the NMF is linear and does not negatively affect the deconvolution. Otherwise, due to the additional constraints of the deconvolution to suppress noise, a nonlinear operation to the data

would be applied before NMF, which can affect spectra on the edge of spatial features.

Supplementary Videos

Video S1:

Unmixed 3D Raman image of a zebrafish eye: Color images (xy) of a 3D hyperspectral image stack (xyzλ) after applying NMF and 3D deconvolution.

Zebrafish:	three days old embryo
Fixed:	4% paraformaldehyde
Embedded:	2% agarose
Slices:	50 á 2048 x 2048 raw spectra
Spectral resolution:	18 cm^{-1}
Irradiance:	870 $\mu\text{W}/\mu\text{m}^2$
Excitation wavelength:	577 nm
Total stack exposure:	14 h 13 min
red:	collagen-like fingerprint
green:	lipid-like fingerprint
blue:	DNA-like fingerprint

References

1. P. G. Pitrone, J. Schindelin, L. Stuyvenberg, S. Preibisch, M. Weber, K. W. Eliceiri, J. Huisken, and P. Tomancak, "OpenSPIM: an open-access light-sheet microscopy platform.," *Nat. Methods* **10**, 598–599 (2013).
2. W. Müller, R. Heintzmann, and M. Kielhorn, "Stabiles Interferometer mit hoher Étendue, insbesondere für bildgebende Fourier-Transformations-Spektroskopie ohne Objektbrasterung," patent DE 10 2014 011 668 A1 (2014).
3. P. R. Griffiths and J. A. de Haseth, *Fourier Transform Infrared Spectrometry*, Second Edi (Wiley, 2007).
4. J. Huisken and D. Y. R. Stainier, "Selective plane illumination microscopy techniques in developmental biology.," *Development* **136**, 1963–1975 (2009).
5. L. Mertz, "Auxiliary computation for Fourier spectrometry," *Infrared Phys.* **7**, 17–23 (1967).
6. M. L. Forman, W. H. Steel, and G. a. Vanasse, "Correction of Asymmetric Interferograms Obtained in Fourier Spectroscopy," *J. Opt. Soc. Am.* **56**, 59–63 (1966).
7. D. B. Chase, "Phase Correction in FT-IR," *Appl. Spectrosc.* **36**, 240–244 (1982).
8. J. Nocedal and S. J. Wright, *Numerical Optimization* (1999), Vol. 43.
9. P. J. Verveer, M. J. Gemkow, and T. M. Jovin, "A comparison of image restoration approaches applied to three-dimensional confocal and wide-field fluorescence microscopy," *J. Microsc.* **193**, 50–61 (1999).
10. F. Soulez, L. Denis, Y. Tourneur, and E. Thiebaut, "Blind deconvolution of 3D data in wide field fluorescence microscopy," 2012 9th IEEE Int. Symp. Biomed. Imaging 1735–1738 (2012).