

# Point Spread Function Measured in Human Skin Using Two-Photon Fluorescence Microscopy

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## Introduction

Scanning fluorescence microscopy using two-photon excitation [1] has become a powerful tool for performing high resolution imaging of optically dense material such as the human skin. For example two-photon microscopy (TPM) has been used for non-invasive skin cancer diagnostics [2] and to study transdermal drug delivery [3]. The human skin is, however, an optical challenge, due to its complex structure and varying optical properties. The cells of the different skin layers alter in size, shape and structure. The optical resolution is therefore not only dependent on the performance of the imaging system, e.g. the excitation wavelength, the numerical aperture of the objective, aberrations and misalignments, but also strongly on the properties of the skin.

One way to correct for these varying optical properties would be to determine how the point spread function (PSF) is changing with imaging depth in human skin. The PSF is generally measured by recording images of subresolution fluorescent beads and then analysing the intensity profiles of the signal from the beads. However, the effective barrier properties of the skin make it difficult to introduce beads and nanoparticles into the tissue [4]. PSF measurements on beads placed on top of skin samples of different thicknesses have been published [5], but these data might not yield a true PSF in epidermis and dermis. Therefore, we have investigated the PSF of subresolution beads injected into skin samples by the use of a syringe.

## Methods

Human skin specimens from Caucasian females, collected as left-overs from breast reduction surgery were used in this work. The specimens were collected in connection to surgery thereafter cut into pieces of 3 x 3 cm and stored in -70 °C for a maximum of four months. For the preparation, they were thawed in room temperature.

Subresolution fluorescent beads (Microscope Point Source Kit, Molecular Probes) of two different colours have been used. The green beads had the excitation/emission maxima at 505/515 nm and the orange beads at 540/560 nm. Both kinds had a diameter of  $0.175 \pm 0.005$   $\mu\text{m}$ . The beads were dissolved in MilliQ water and sonicated for five minutes. The solution was injected into the skin sample using a syringe (needle diameter 0.40 mm). The prepared skin sample was mounted on a microscope slide in an imaging chamber.

The two-photon microscope used for imaging was a Bio-Rad Radiance 2100MP Rainbow system built on an inverted Olympus IX71 microscope. The excitation light was provided by a pulsed fs Ti:sapphire Tsunami laser, tunable in the range 700 – 1000 nm and the excitation wavelength was set to 780 nm. A 40x/0.8 water-immersion objective with a numerical aperture of 0.8 and a working distance of 1.7 mm was used.

The PSF of the individual beads were measured by obtaining their lateral and axial profiles in ImageJ (Wayne Rasband, National Institute of Mental Health) and fitting a Gaussian function to the profiles in SigmaPlot (Systat Software Inc.(SSI), USA).

## Results

Subresolution beads were injected into human skin and imaged using TPM in order to obtain the PSF at different tissue depths. Z-series, with a step size of 0.1  $\mu\text{m}$ , of the beads were taken at depths between 0 - 35  $\mu\text{m}$ . The results are presented as the full width at half maximum (FWHM). Figure 1 show the axial and lateral slices through the centre of a green bead where the centre of the bead is located at 2.4  $\mu\text{m}$ . As expected, the axial resolution is worse than the lateral, resulting in an image where the bead appears elongated.

The PSF measurements obtained at varying depths in the skin specimens are presented in Table 1 and 2. The values of the lateral FWHM are relatively independent of tissue depth, while the axial FWHM is increasing with increased imaging depth. There were no major differences in the PSFs between green and orange beads, indicating that the TPM-PSF is predominantly determined by the excitation wavelength rather than the wavelength of the emission.

Based on previously empirically determined relations (Eq 1, 2) between excitation wavelength, numerical aperture and refractive index for the TPM-PSF [6], the lateral and axial values using our set up can be estimated to:  $\text{FWHM}(r) = 0.37 \mu\text{m}$  and  $\text{FWHM}(z) = 0.94 \mu\text{m}$ , where  $\text{FWHM} = \omega 2\sqrt{\ln 2}$ . The calculations are based on the refractive index of water,  $n = 1.33$ , since a water immersion objective was used. The measured values of the lateral FWHM are quite close to this calculated value, while the axial values differ more. This implies a larger refractive index mismatch and more pronounced aberrations in the lateral direction.

$$\omega_{xy} = \frac{0.325 \cdot \lambda}{\sqrt{2} \cdot NA^{0.91}} \quad (1)$$

$$\omega_z = \frac{0.532 \cdot \lambda}{\sqrt{2} \left( n - \sqrt{n^2 - NA^2} \right)} \quad (2)$$

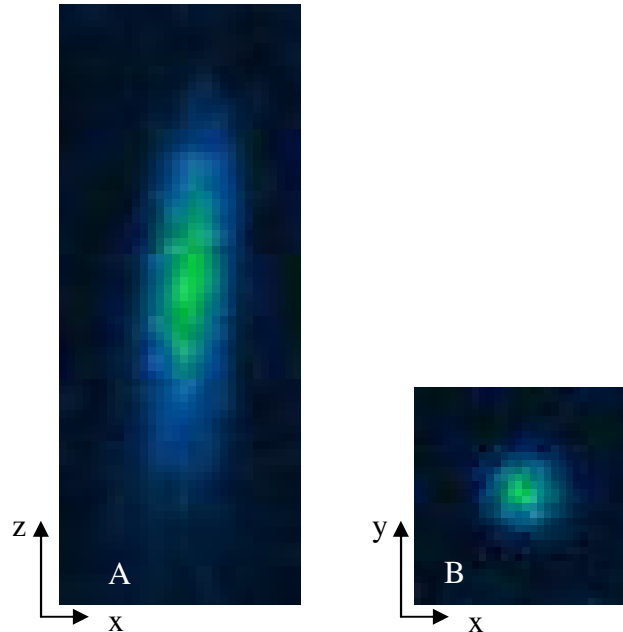


Figure 1. The TPM-image (pseudo-colour intensity code) of a green bead in axial direction (A) and lateral direction (B). The axial slice (A) covers the distance 0 — 5  $\mu\text{m}$  and the lateral slice (B) is at the distance 2.4  $\mu\text{m}$ .

Table 1. The FWHM-values for the green beads (filter 450-530nm)

Depth* [μm]	Depth range for z-stack [μm]	FWHM(r)[μm]	FWHM(z) [μm]
2	0-5	0,34 ± 0,04	1,3 ± 0,08
25	23-28	0,37 ± 0,07	1,2 ± 0,01
27	23-28	0,39 ± 0,04	1,4 ± 0,3
29	25-35	0,39 ± 0,13	1,6 ± 0,2

\* centre of bead

Table 2. The FWHM-values for the orange beads (filter 570LP nm)

Depth* [μm]	Depth range for z-stack [μm]	FWHM(r) [μm]	FWHM(z) [μm]
2	0-5	0,30 ± 0,01	1,4 ± 0,1
33	25-35	0,37 ± 0,02	1,6 ± 0,2

\* centre of bead

## Conclusions

We have shown that by injecting subresolution beads in skin samples by the use of a syringe, the internal PSF could be measured at different depths. The measured values of the lateral FWHM ( $0.37 \pm 0.02$ ) lie quite close to the calculated value ( $0.37 \mu\text{m}$ ) obtained from empirically determined equations [6]. The axial values of FWHM on the other hand, differ substantially from the calculated. This result indicates that the mismatch of refractive index and aberrations have larger effects in the lateral direction. Future work will be focused on obtaining more measurements at different depths and to go deeper in the skin. Alternative ways of introducing the beads in the skin are also of interest.

## References:

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