APPLICATION OF STRUCTURED ILLUMINATION MICROSCOPY TO CORNEAL IMAGING

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Introduction

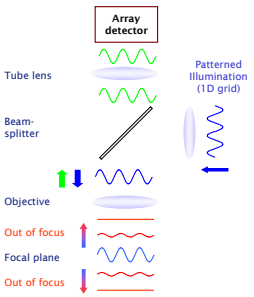
Microscopic studies of corneal structures have always been a challenge for ophthalmologists and researchers. The inherent transparency of the cornea and the strong out of-focus background are the main obstacles for achieving high-contrast images. Traditional approaches based on the confocal principle do not yet fully satisfy all the requirements of resolution, acquisition speed and simplicity, which are essential for in-vivo research.

Structured illumination microscopy

Structured illumination microscopy allows optical sectioning to be performed on a wide-field microscope system [1]. Image acquisition is potentially faster, as optical sections of the whole field of view are obtained recording three or four images, rather than scanning every point in the field of view. In practice, a high spatial-frequency (ν) grid-pattern is projected onto the sample at different phases and the image is reconstructed using the following algorithm for four images at equidistant phases:

$$I_{in-focus} = \frac{1}{2}\sqrt{(I_1 - I_3)^2 + (I_2 - I_4)^2}$$

where $I_n = I_{widefield} + I_{in-focus}\cos\left(\frac{2\pi}{\nu}x + \phi_n\right)$ are the recorded images corresponding to each phase ϕ_n



Data quantification in four phase SIM

There are three main sources of noise in SIM:

- Shot-noise associated to the detection of each phase image [2]
- Noise associated to the reconstruction of the in-focus image
- Noise associated to phase inaccuracy: Phase inaccuracies can be accounted for using a least-squares algorithm [3]

} **Non-gaussian statistics**

If a linear and well-behaved wide-field detector (CCD) is used, each pixel in a phase image is given by

$$I_n = \frac{\text{number of photons}}{K} \quad \text{with} \quad \sigma_{I_n}^2 = I_n/K + \sigma_{RO}^2$$

where σ_{RO}^2 is the variance of the read-out noise.

The reconstructed image follows a Rician distribution, with a mean that is not the actual in-focus value

$$p(I_{reconstructed}) = \frac{I_{reconstructed}}{\sigma_s^2} e^{-\frac{I_{reconstructed}^2 + I_{in-focus}^2}{2\sigma_s^2}} I_0\left(\frac{I_{reconstructed} \cdot I_{in-focus}}{\sigma_s^2}\right)$$

Mean of the Rician distribution: $\mu = \sigma_s\sqrt{\frac{\pi}{2}} \cdot L_{1/2}\left(-\frac{I_{in-focus}}{2\sigma_s^2}\right)$

where $I_0(z)$ is a modified Bessel function of the first kind,

$L_{1/2}(z)$ a Laguerre polynomial, and $\sigma_s^2 = \frac{I_{widefield}}{2K} + \frac{\sigma_{RO}^2}{2}$

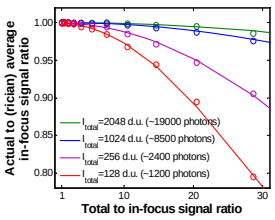


Fig 1. Ratio of the actual value of an in-focus pixel in an image to the expected value after SI reconstruction as a function of the ratio of total to in-focus signal. The lines show the theoretical Rician dependence for four different levels of total signal (in digital units); the circles are the values after reconstruction of 5000 simulated images recorded using a 4 phase SI algorithm and a CCD with K=9.3 photons·d.u.$^{-1}$ and σ_{RO}=2.1 d.u.

Noise in four phase SIM

SIM vs. perfect confocal system

SIM	Confocal
· MTF-fall off: 70%	· Pinhole: 0.68 Airy
· Detective QE: 70% (CCD)	· Detective QE: 30% (PMT)
· 250x250 pixels in 30 ms (C-link)	**· 150x150 pixels in 24 ms (1 µs dwelling time)**

A large background affects SI performance, but…

- SI acquisition is potentially faster than confocal acquisition when the background is low
- SIM may allow images with higher resolution to be obtained [4]

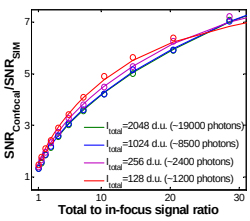
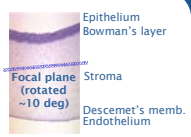


Fig 2. Ratio of the SNR after confocal acquisition to the SNR after 4-phase SI acquisition and reconstruction for the same number of photons delivered to the sample. Detective efficiencies for each system may be found above. The lines show the theoretical Rician ratio for four levels of total signal (in digital units); the circles are the values after reconstruction of 5000 simulated images recorded using a 4 phase SI algorithm and a CCD with K=9.3 photons·d.u.$^{-1}$ and σ_{RO}=2.1 d.u.

SIM of the cornea

Non-contact microscopic imaging of the unstained cornea using SIM [5] has been demonstrated. Cells within the corneal stroma and endothelium could be distinctly identified in the following samples.

- Resectioned chick corneas.
- Enucleated porcine eyes.



H&E resection of a cornea

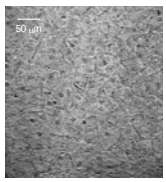


Fig 4. SI sectioned image of the corneal endothelium of an enucleated porcine eye. The characteristic hexagonal cells are distinctly identified (x20, NA 0.40).

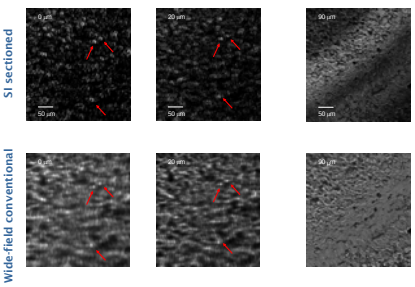
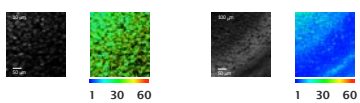


Fig 3. SI sectioned and widefield conventional images of a resectioned chick cornea at different depths within the stroma (first two columns) and the endothelium (last column) (x20, NA 0.40).



Theoretical total signal to in-focus signal ratio

· Cell membrane:	1.45	Stroma:	1.38
· Cytoplasm:	1.37	Aqueous humour:	1.34
· Air (non-contact):	1	T/S ratio:	2<T/S<40
· Immersion gel:	**1.35**	**T/S ratio:**	**1.5<T/S<2.5**

Non-contact imaging of the stroma shows a high background level which compromises the SNR; non-contact imaging of the endothelium shows a low background level which results in a high SNR.

Provided that the reflections from the optics inside the microscope can be beaten, we can foretell that immersion gel mediated SIM may perform comparably to confocal microscopy, with potential advantages in speed and resolution.

References

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