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Structured illumination microscopy (SIM)

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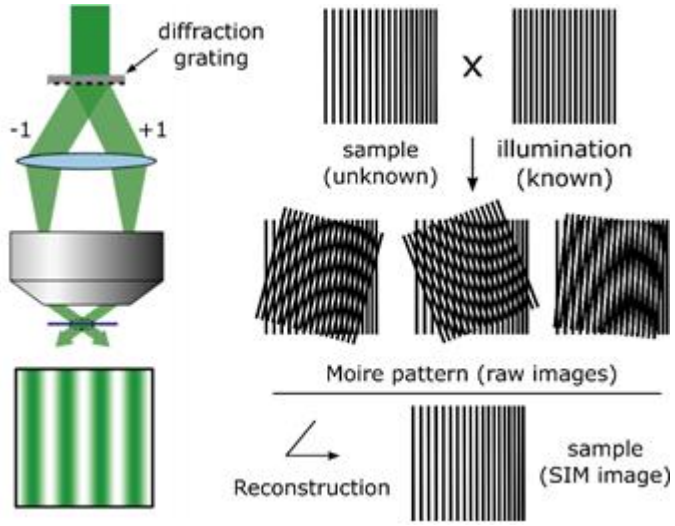

Fig 1. Principle of SIM technique. (from andor.oxinst.com)

Aims of the practical

- Comparing 2 different experimental setups for SIM
- Understanding the importance of polarization control to produce good modulation contrast of the illumination pattern
- Reconstruction of sample images from raw SIM data
- Identification of the main sources of artefacts in SIM and methods to minimize through optimizing sample preparation

Background

The SIM method takes advantage of low frequency beats which occur in an image when a fluorescent sample is excited with a striped excitation pattern in an epifluorescence microscope (see figure above). In the original 2D-SIM approach, the illumination pattern consists of lateral stripes of a single frequency with a stripe distance close to the resolution

limit of the optical system (i.e., ~200 nm with a 1.4-NA oil immersion objective). The stripes are generated by the interference of two beams of light, which are positioned to enter the objective's back-focal aperture close to opposite edges.

The illumination pattern interacts with the sample, and the resulting low frequency beat patterns in the fluorescent image encodes information from the sample than is usually not observable in conventional microscopy because of diffraction. The beat patterns are also called Moiré fringes. If the illumination pattern is known, the underlying sample pattern can be calculated from the Moiré fringes. In practice, multiple images have to be collected with the stripe pattern laterally shifted to different phase positions (typically three within the sinusoidal cycle of one stripe period; step size: $2\pi/3$) and rotated (typically in three angles with steps of 60°, respectively).

For the super-resolution reconstruction, the data are Fourier-transformed (by fast Fourier transform (FFT)) to convert them to a spatial frequency representation. The information acquired at different phase positions of the SIM stripes produces a set of linear equations to separate the relative contributions from the lower frequencies (equivalent to conventional wide-field imaging) and higher frequencies (the super-resolution information), a step that is also referred to as 'band separation'. The higher frequencies are extracted and shifted to the correct position in frequency space, and different angles are combined to produce a nearly isotropic restoration of the image in the xy dimension. For super-resolution image generation, the data is then inverse Fourier-transformed into real-space information, thus producing a resolution-doubled image. Importantly, recombining the different spatial frequency components requires good knowledge of the amplitude at which the optical system transfers this information. This is usually extracted from the system's optical transfer function (OTF). Only when the OTF and the sample properties during acquisition are reasonably matched will the reconstruction algorithm produce valid results, without notable artefacts.

Another critical factor is the amplitude of the stripe modulation in the collected images. The stripes enable the shifting of high spatial frequency information to lower, detectable frequencies. The modulation contrast of these stripes is directly linked to the amount of retrievable high-frequency information. The resolution improvement achieved in SIM relies on both a fine spacing and a high-modulation depth for the excitation light pattern. Therefore, a coherent light source (i.e., a laser) capable of introducing destructive interference in the pattern is required for maximum contrast and thus best performance. Incoherent light sources (such as LEDs) can be used only when compromises in resolution improvement or SNR are acceptable.

Further reading prior to the practical (required reading in bold)

1. Gustafsson, M. G. L. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J. Microsc.* **198,** 82–7 (2000).
2. Jost, A. & Heintzmann, R. Superresolution Multidimensional Imaging with Structured Illumination Microscopy. *Annu. Rev. Mater. Res.* **43,** 261–282 (2013).
3. **Heintzmann, R. & Huser, T. Super-Resolution Structured Illumination Microscopy. *Chem. Rev.* 117, 13890–13908 (2017).**

Questions

- Conceptual questions
    - Why does 2D SIM require a total of 9 raw images to reconstruct one high resolution image?
    - What methods can be used to create the illumination pattern required for SIM?
    - Why is the improvement of normal SIM limited to 2x the resolution of conventional epifluorescence microscopy? Can you think of ways of going beyond this resolution limit?
    - What are the advantages of SIM in addition to the resolution enhancement?
    - What are the disadvantages of SIM?
    - What are main sources of artefacts in SIM imaging?
    - Which variants of SIM exist?
- Practical questions
    - Discuss the need for controlling the polarization state of the illumination light, and how this can be achieved in practice.
    - What are important aspects of sample preparation?
    - Could you use a supercontinuum source for illumination in SIM? Discuss!
    - What are biological applications of SIM?

Outline of the practical

- Image ideal/non-ideal samples
    - 2 different mounting media
    - 2 different background signals
- Vary pockels cell values to see impact of polarization on image quality
- Reconstruction and comparison of images

Safe use of lasers in this practical

Laser light poses a potential blinding hazard. This practical must be done under supervision of an experienced user of the localisation microscope, and the following key considerations must be followed.

1. The lasers used in this microscope are visible lasers of **Class 3B**. Class 3B lasers pose an eye hazard only if the beam is viewed directly. Hence, it should not be possible to directly view the laser beam (or to view a mirror used to steer the beam) when the laser is being operated at anything above a low power setting (1 mW is a suitable low power setting, since this is a low power setting that does not pose an eye hazard).
2. Laser beams should be fully enclosed during normal use, to prevent any chance of direct exposure to laser light.
    a. The light source enclosure should be closed during the actual measurement.

   b. The microscopy specimens should be covered with a light-proof black box before the illumination shutter is opened.
3. **Laser beams should be set to a low power (less than 5 mW) before any part of the laser beam path is uncovered for any reason** (for example, for alignment).
4. A particular hazard with the TIRF system is that the spatial light modulator (SLM) acts as a controllable reflective surface that can deflect laser light in any direction. If the illumination enclosure of the SIM system is open for alignment, then there is a risk that specular reflections of the illumination beam might be deflected from the SLM in unpredictable directions. Therefore it is particularly important that the illumination enclosure is covered during normal use of the system, and that if this part of the system is open for alignment or system development then the laser beam power must be set to less than 5 mW.


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