Chapter 2

LAG SIM: A versatile, user-friendly structured illumination microscope

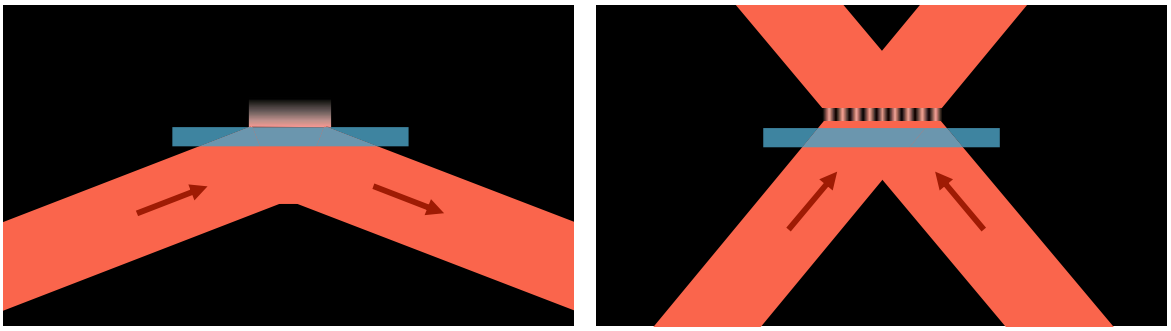
2.1 Introduction

2.1.1 Background to SIM

Widefield epifluorescent microscopes use a uniform field of light to illuminate a labelled sample [14, *ch. 2*]. Fluorescent emission light is then emitted from any fluorescent molecule located within the microscope's field of view [56]. In this type of microscope, fluorescent molecules above and below the focal plane of the lens will also receive illumination light and fluoresce, causing an out-of-focus blur on the image [57].

Out-of-focus light can be removed by placing a pinhole in the excitation and emission path, and scanning the small spot of light across the sample to build up an image one pixel at a time. This technique, invented in 1955 and called confocal microscopy, physically blocks out-of-focus light, making 3D reconstructions of samples possible [28]. The cost of this optical sectioning is a slow acquisition speed due to the nature of a point-scanning technique.

A faster way of removing out-of-focus light can be achieved with total internal reflection fluorescence (TIRF) [58, *ch. 21*]. In this scheme light is projected into the outer edge of the back-aperture of a specialised lens, such that it emerges from the lens steeper than the critical angle required for reflection, rather than refraction, from a glass interface. Although this means that no light passes through the microscope slide, as shown in Figure 2.1a, energy from the evanescent field is able to couple into molecules close to the coverglass, inducing fluorescence [59]. TIRF illuminates molecules within 100 nm of the coverglass without obscuration by out-of-focus light, with the disadvantage that details deeper into the sample cannot be observed.



(a) **(b)**

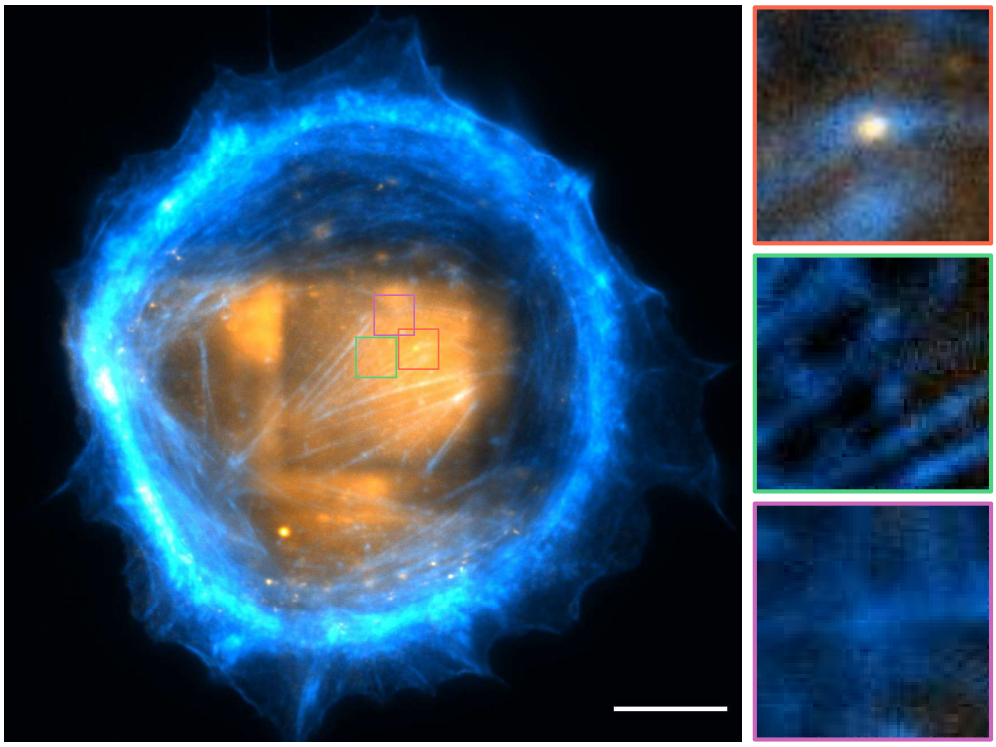
Figure 2.1: (a) shows a TIRF illumination scheme, where light intercepts the cover glass at an angle steeper than the critical angle for total internal reflection. The evanescent wave created can excite fluorphores in the closest 100 nm to the coverglass, decaying exponentially in intensity. (b) shows the generation of a structured illumination pattern by the interference of two beams. This can be used to computationally reconstruct an optically sectioned image, as shown in Figure 2.2. Diagrams are not to scale.

Sheppard and Wilson showed that optical sectioning can be achieved computationally if the illumination light is modulated with a structured pattern [56, 60]. Sinusoidal illumination can be produced by two coherent beams of light generated by a diffraction grating, which interfere at the focal plane of the objective lens as shown in Figure 2.1b. The resulting image I is described by Equation 2.1, where W is the equivalent widefield image without structured illumination, m the modulation depth, t the modulation frequency, and x a lateral spatial dimension in the sample plane.

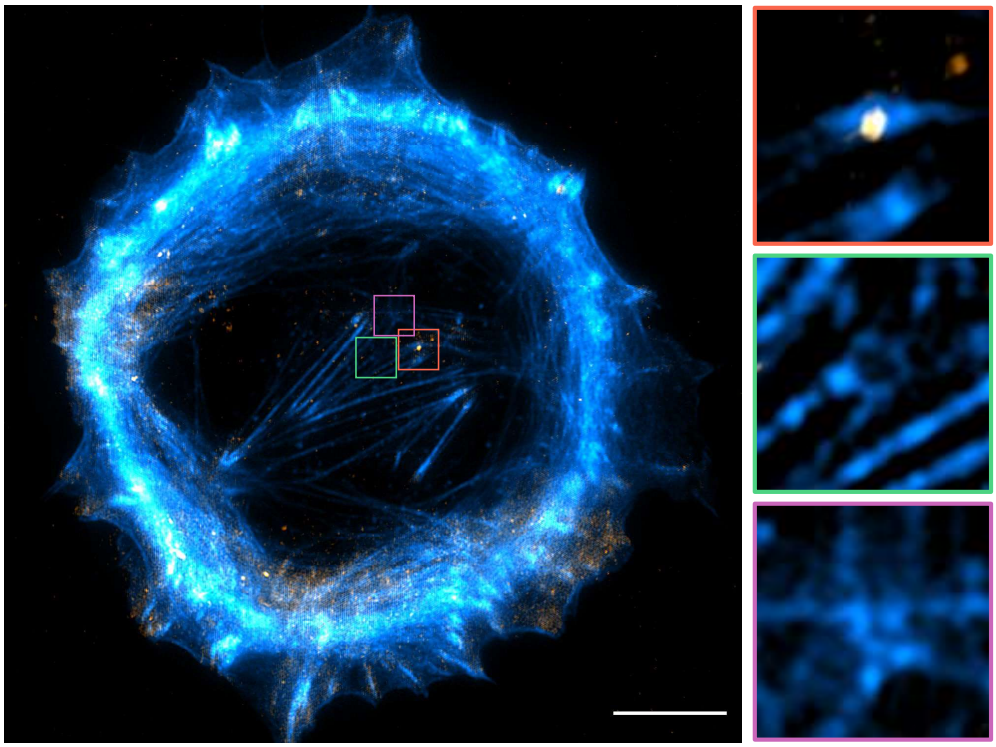
$$I_i = W\left(1 + m\cos\left(tx + \phi_i\right)\right) \tag{2.1}$$

Since the sinusoidal illumination pattern is only generated in the focal plane of the objective lens, extracting the Wm component from Equation 2.1 constructs an image I_R in which the unmodulated out-of-focus light has been removed. Proofs 2.1 and 2.2 show that this requires the illumination to be stepped through three equally-spaced phases $\phi_i = \left\{0, \frac{2\pi}{3}, \frac{4\pi}{3}\right\}$, with an image I_i captured at each step $i = \{1,2,3\}$. The proofs verify that either the square law detection of Equation 2.2 or the homodyne detection shown in Equation 2.3 can be used to extract just the Wm term [60], multiplied in each case by a constant scaling factor.

If the acquired images are simply summed together, the result $I_1 + I_2 + I_3$ is W, the image that would be obtained from a widefield microscope. Figure 2.2a shows this sum for an image of the herpes simplex virus infecting a human foreskin fibroblast (HFF) cell. When the raw images I_i are reconstructed with the square-law detection of Equation 2.2, however, Figure 2.2b shows the effective removal of out-of-focus light.



(a)



(b)

Figure 2.22: (a) shows actin labelled in MEF cells, where out-of-focus light blurs details in the actin. Utilising TIRF-SIM, shown in (b), enhances resolution and removes out-of-focus light. Inset images show various stages of actin development, with a dense cluster of actin (inset in red box) forming into a ring structure (inset in green box) which acts as a source for new filament growth (inset in purple box). Cells were prepared for imaging by Anchal Chandra. Scalebars are 10 µm long, and inset images are 3.2 µm squares.

2.6.5 Resolution-enhanced optical sectioning of brain-slice tissue imaging for measuring the Node of Ranvier

Neurons in the brain communicate at junctions known as synapses, which are formed where an axon from one neuron meets a dendrite from another [102]. The axons, coloured yellow in Figure 2.23, form long, thin projections, and can be compared to electrical wires carrying signals across the brain to control bodily functions such as sensing, movement, and conciousness.

Figure 2.23 shows axons coated in a fatty sheath known as myelin [102]. This protects the axon from interference with other nearby axons, and increases the transmission speed of electrical signals as action potentials jump from one gap in the myelin sheath - known as a Node of Ranvier - to the next in a process called saltatory conduction [103]. Diseases which cause injury to the myelin sheath are associated with motor neuron disorders such as multiple sclerosis and leukodystrophy [104].

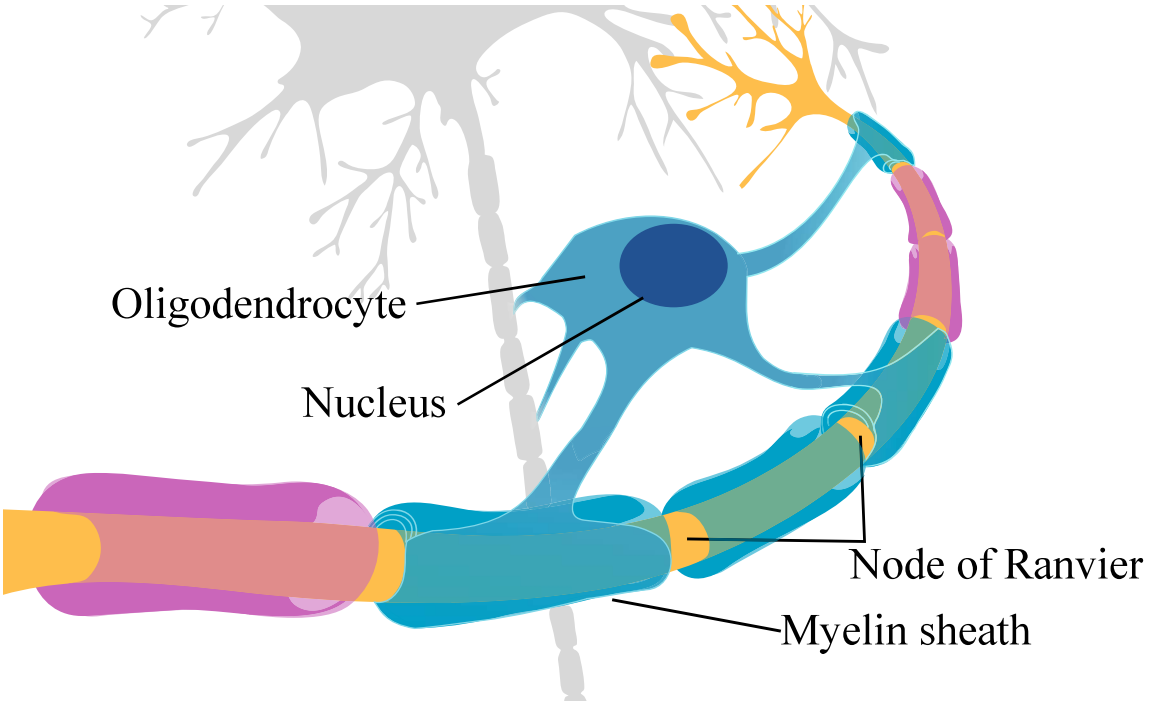


Figure 2.23: Diagram of a neuron, adapted from Wikipedia [105]. A neuron, coloured in yellow, has long wire-like projections called axons. Axons are covered by a fatty coating known as the myelin sheath, which protects it from interference from neighbouring axons and increases the transmission speed of signals. The myelin sheath is part of a different brain cell type called an oligodendrocyte, shown here in blue. The gap between two myelin sheaths is known as the Node of Ranvier.