9
Super-Resolution Microscopy: Interference and Pattern Techniques
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9.1 Introduction

In many scientific fields such as biology, medicine, and material sciences, microscopy has become a major analytical tool. Electron microscopy (EM) with nanometer resolution, on the one hand, and light microscopy with a broad applicability, on the other hand, allowed for groundbreaking scientific achievements. Even though EM delivers unmatched resolution, light microscopy has never lost its relevance. Because of the vast propagation of fluorescent labeling techniques in recent years, the method of fluorescence microscopy has actually become one of the most important imaging techniques in the life sciences.

However, the intrinsically limited resolution of standard fluorescence microscopy compared to nonlight-optical methods is still a major drawback as many biological specimens are of a size in the nanometer to micrometer range. Therefore, the standard light-microscopic resolution defined by Rayleigh (1896) (Chapter 2) is often not sufficient to resolve the objects of interest.

Over last years, different techniques denoted as super-resolution fluorescence microscopy have been established to compensate for the deficiency of low spatial resolution. These approaches use fluorescence excitation because this circumstance allows – in combination with other techniques – access to high-resolution object information.

These super-resolution methods (i.e., 4Pi (Cremer and Cremer, 1978; Hell and Stelzer, 1992; Hell et al., 1994; Hänninen et al., 1995 stimulated emission depletion (STED) (Chapter 10), SIM/PEM (Gustafsson, 2000; Heintzmann and Cremer, 1999), and localization methods (Chapter 8)) seemingly break the conventional resolution limit. However, it should be noted that the fundamental resolution limit is not broken directly. The super-resolution methods are based on conditions differing from the assumptions of Rayleigh. Rayleigh’s conclusions for self-luminous (i.e., fluorescent) objects do not consider spatial or temporal variations of the light intensity. Basically, all super-resolution techniques depend on juggling with the fluorescence excitation or emission.
In this chapter, we describe two wide-field methods that apply interference of the excitation light to make high-resolution object information accessible in detail. These are the method of structured illumination microscopy (SIM) (also referred to as patterned excitation microscopy, PEM) and the method of spatially modulated illumination (SMI).

9.1.1 Review: The Resolution Limit

Regarding imaging systems, it is obvious that the rendering power (i.e., the capability to transmit structural information) is always limited to some degree. To describe the rendering power of an imaging system, the term resolution is employed. However, the term resolution is ambiguously used and there exist different scientific definitions for the resolution power of a system.

A commonly used resolution measure is the definition provided by Lord Rayleigh, which is described in Chapter 2. Rayleigh’s definition is based on the point spread function (PSF) of an optical system (Section 2.3.3).

The PSF or Airy function resembles the image of an ideal pointlike object. The Rayleigh distance defining the resolution is the distance between the maximum and the closest zero point of the PSF.

It is given by

$$d_{\text{Rayleigh}} = 0.61 \frac{\lambda}{n \sin \alpha}$$

(9.1)

In Chapter 2, the optical transfer function (OTF) is introduced. The OTF is the Fourier transform $F$ of the PSF.

$$F[\text{PSF}(x,y)] = \text{OTF}(k_x, k_y)$$

(9.2)

When the PSF is the same for every position in the object plane, every point of the object is broadened by the same PSF. Therefore, the imaging process can be represented by a convolution:

$$A'(x,y) = A(x,y) \otimes \text{PSF}(x,y)$$

(9.3)

Here, $A'(x,y)$ is the image and $A(x,y)$ is the object. The convolution theorem states that convolution in position space corresponds to multiplication in frequency space and vice versa. Hence, the imaging process can be described in Fourier space by

$$F[A'] = F[A] \times \text{OTF}$$

(9.4)

The PSF is band limited, which means that the OTF is zero for high frequencies beyond a cut-off frequency $k_{\text{cut-off}}$:

$$\text{OTF}(k) = 0 \quad \text{for} \quad |k| \geq k_{\text{cut-off}}$$

(9.5)

All object information beyond this cut-off frequency is filtered out by the OTF and is therefore missing in the image. The lack of high frequencies in the image is the reason for the limited resolution. In Figure 9.1, the cut-off frequency is shown in three dimensions (3D).
The cut-off frequency can be used as a measure for the resolution of an optical system, as an alternative to the Rayleigh definition.

A resolution definition referring to the object’s frequency is the Abbe definition (Abbe, 1873) of resolution. Abbe considered a fine grating placed on a microscope. The finest, yet resolvable, pattern (with grating distance $d_{\text{Abbe}}$) would define the resolution of the microscope. The calculation yields

$$d_{\text{Abbe}} = \frac{\lambda}{2n \sin \alpha} \quad (9.6)$$

The corresponding maximum transmittable frequency (i.e., cut-off frequency) is given by $k_{\text{cut-off}} = 2\pi/d_{\text{Abbe}}$.

In our case, both definitions $d_{\text{Rayleigh}}$ and $d_{\text{Abbe}}$ are proportional and yield to similar values. In the following paragraphs, ways to circumvent this resolution limit are discussed, which are based on a manipulation of the illumination conditions.

9.2 Structured Illumination Microscopy (SIM)

In SIM, the object is illuminated with a periodic illumination pattern.

This periodic excitation pattern is used in order to manipulate the object’s spatial frequencies (Heintzmann and Cremer, 1999; Gustafsson, 2000) as described in the following sections. In SIM, the pattern is projected through the (single) objective lens and no additional devices or mechanisms are used to illuminate the object from the opposite side.

From another point of view, it can be said that the pattern is generated by an interference of a number of coherent beams proceeding from the objective lens, meeting in the object plane (Figure 9.2).
The periodic pattern can be of arbitrary shape, but typically the modulation of the pattern is only one dimensional in the focal plane ($x - y$-plane) as is the case with the patterns shown in Figure 9.2. Two methods to spatially modulate the excitation intensity in SIM are common: two-beam interference (also denoted by fringe projection) and three-beam interference (grid projection). Examples for optical setups generating the coherent beams are given in Section 9.2.6.

In the three-beam interference case, one light beam propagates along the optical axis and two beams span an angle with the first beam. One of the other beams propagates at an angle $\alpha$ to the optical axis and the other one at the angle $-\alpha$. All three beams span a common plane (interferometer plane). The resulting interference pattern has a modulation along one direction in the focal plane and also a modulation along the optical axis.
9.2 Structured Illumination Microscopy (SIM)

The other method works with only two beams and corresponds to the three-beam case with a blocked central beam along the optical axis. The corresponding interference pattern exhibits only a modulation along the focal plane \((x - y)\)-plane).

In the following, the image formation process and, thus, the resolution improving effect of SIM will be shown using the more simple method of two-beam interference.

9.2.1 Image Generation in Structured Illumination Microscopy

In this section, a mathematical description of the image generation process in SIM is developed.

As mentioned in Section 9.1.1 and in Chapter 2, the imaging process of a wide-field microscope can be described by

\[
A'(x,y) = A(x,y) \otimes \text{PSF}(x,y) \quad (9.7)
\]

where \(A'\) is the image and \(A\) the object. The PSF is the impulse response blurring the image. The object \(A\) is the fluorescence light distribution in the object plane.

When fluorescence saturation is insignificant (under usual fluorescence microscopy conditions), the object distribution is proportional to illumination intensity \(I_{\text{ill}}(x,y)\) and fluorophore distribution \(\rho(x,y)\):

\[
A(x,y) = \rho(x,y) \times I_{\text{ill}}(x,y) \quad (9.8)
\]

In conventional wide-field microscopy, the illumination intensity is constant and the object is essentially the fluorophore density.

In SIM, however, the illumination is spatially variant and this variation is carried through into the image. For the case of two-beam illumination (Figure 9.2a), the illumination distribution is given by

\[
I_{\text{ill}}(x,y) = I_0 \left[ 1 + m \cos \left( k_G x + k_G y + \varphi \right) \right] \quad (9.9)
\]

or

\[
I_{\text{ill}}(r) = I_0 \left[ 1 + m \cos \left( k_G r + \varphi \right) \right] \quad (9.10)
\]

in vector notation with the position vector \(r\).

The modulation strength \(m\) is a scalar value between 0 and 1. For \(m = 1\), the distribution has a minimum of zero and a maximum of \(2I_0\). The intensity modulation takes place in the \(x - y\)-plane. To express the modulation in the 2D space, the \(k\)-vector of the grating \(k_G\) with \(|k_G| = 2\pi/G_{\text{SIM}}\) is used, where the grating constant \(G_{\text{SIM}}\) is the period of the modulation. \(k_G\) points in the direction of the modulation (i.e., it is perpendicular to the stripes of the pattern and pointing along the object plane \((x - y)\)-plane)). The lateral position of the illumination pattern is determined by the phase \(\varphi\).

Substituting Eq. (9.8) in Eq. (9.7) yields

\[
A'(r) = \left[ \rho(r) \times I_{\text{ill}}(r) \right] \otimes \text{PSF}(r) \quad (9.11)
\]
Now, the image information in Fourier space is given by
\[
\sim A = FT [A^\prime] = FT \left[ \rho \times I_{\text{ill}} \right] \times \text{OTF}
\] (9.12)

The multiplication of \( \rho \) and \( I_{\text{ill}} \) transforms into a convolution because of the convolution theorem:
\[
\sim A = \tilde{\rho} \otimes \tilde{I}_{\text{ill}} \times \text{OTF}
\] (9.13)

The Fourier transform of the illumination pattern (Eq. (9.9)) is
\[
\tilde{I}_{\text{ill}} (k) = \frac{1}{2\pi} \times I_0 \left[ 1 + m \cos (k_G r + \varphi) \right] e^{-i(kr)} \, dr
\] (9.14)

Using the identity
\[
\cos x = \frac{1}{2} (e^{ix} + e^{-ix})
\] (9.15)

FT\((I_{\text{ill}})\) becomes
\[
\tilde{I}_{\text{ill}} (k) = \frac{1}{2\pi} \times I_0 \left( e^{-ikx} + \frac{m}{2} e^{-i(kx-k_G x)} e^{i\varphi} - \frac{m}{2} e^{-i(kx+k_G x)} e^{-i\varphi} \right) \, dr
\] (9.16)

Expanding this equation yields
\[
\tilde{I}_{\text{ill}} (k) = \frac{1}{2\pi} \times I_0 \left[ \delta (k - \alpha) + \frac{m}{2} e^{i\varphi} \delta (k - k_G) + \frac{m}{2} e^{-i\varphi} \delta (k + k_G) \right]
\] (9.19)

One delta function is located at the origin and two at the reciprocal period of the illumination pattern.

In the Fourier-transformed image \( \sim A^\prime \), the convolution of these delta functions with the Fourier-transformed object distribution leads to a sum of copies of the object information shifted by the positions of the delta functions. Each copy is multiplied by its corresponding complex coefficient:
\[
\sim A^\prime (k) = \text{OTF} (k) \times I_0 \frac{1}{\sqrt{2\pi}}
\times \left[ \left( \hat{\rho} (k) + \frac{m}{2} e^{i\varphi} \hat{\rho} (k - k_G) + \frac{m}{2} e^{-i\varphi} \hat{\rho} (k + k_G) \right) \right]
\] (9.20)

How far the single additional copies are shifted in Fourier space is defined by the angle \( \alpha \) and the wave vector \( k \) of the incident light waves.

Note the low-pass filter property of the OTF (during image formation, the OTF is multiplied by \( FT[\rho \times I_{\text{ill}}] \)). As the frequencies of the additional copies of the image are shifted, a frequency region of previously irresolvable frequencies beyond the cut-off limit \( k_{\text{cut-off}} \) of these copies is shifted into the area of frequencies that
are transmitted by the OTF. This frequency information is superposed with the information of the other copies and located in the frequency region imaged by the microscope. Therefore, it has to be separated and shifted back to its appropriate position after image acquisition. The separation and reconstruction process will be discussed in Section 9.2.2.

When it is possible to separate the shifted information in the acquired image, the information can be shifted back to its original position. It is apparent that now the frequency region of the image spans further in the direction of the modulation of the illumination pattern compared to the original image. Therefore, the resolution has been enhanced by this process as higher frequency information is transmitted into the image. The factor of resolution improvement is given by the factor by which the size of the frequency region of the image is expanded.

The size of the resulting frequency region depends on the grating distance of the illumination pattern. To calculate the maximum possible resolution improvement by SIM, the minimum possible grating distance has to be taken into account. It is reached when the two incident coherent excitation beams with the vacuum wavelength $\lambda_{\text{ex}}$ span the maximum angle. Therefore, the minimum grating distance is given by

$$G_{\text{SIM min}} = \frac{\lambda_{\text{ex}}}{2n \sin (\alpha_{\text{max}})} = \frac{\lambda_{\text{ex}}}{2 \text{NA}} = d_{\text{Abbe}}$$

which is the Abbe definition for the minimum grating distance of a yet resolvable grating in the object plane (for transmitted light microscopy).

In fluorescence microscopy, the wavelength of the emission light is close to that of the excitation light ($\lambda_{\text{em}} \approx \lambda_{\text{ex}}$). Hence, the reciprocal grating period of the smallest possible illumination pattern approximates the cut-off frequency $k_{\text{cut-off}}$. The delta functions of the Fourier-transformed illumination pattern are located at the positions $-2\pi/G_{\text{SIM}}$ and $+2\pi/G_{\text{SIM}}$. Therefore, the origins of the copies of Fourier-transformed information are shifted to the cut-off limit (Figure 9.3b). When these copies are separated and shifted back, the region of accessible information in Fourier space is twice as large as in the standard microscopy case. Thus, the resolution improvement in linear SIM can reach a factor of 2.

The term *linear* is used because in this case, the dependency of the fluorescence emission intensity is considered to be linear to the excitation intensity.

This assumption is true only for low-illumination conditions, where saturation and photobleaching effects can be neglected (which is the case under usual microscopy conditions). Saturation and photobleaching effects result in a decreasing gradient of the emission response with increasing excitation intensity. It has been shown (Heintzmann, Jovin, and Cremer, 2002; Gustafsson, 2005) that this nonlinear effect can be used to increase the resolution of SIM beyond the factor of 2 when the magnitude of the nonlinearity is maximized by the application of appropriate setup conditions (e.g., special fluorochromes, matched excitation intensities). In this case, the effective fluorescence pattern that is multiplied with the fluorochrome distribution contains the periodic illumination intensity pattern and additionally higher harmonics of the fundamental pattern. In Fourier space,
the higher harmonics shift the image information by the double, triple, or more amount of the shift by the fundamental pattern.

The twofold resolution improvement in linear SIM yields a lateral resolution of $\sim 100$ nm (Heintzmann and Ficz, 2007), whereas wide-field- and confocal microscopy achieve $\sim 230$ and $\sim 180$ nm lateral resolution, respectively (Pawley, 2006).

However, when an illumination pattern with modulation in only one direction in the optical plane (i.e., the plane of focus) is used, the resolution improvement is anisotropic and only maximal in the direction of the modulation (Figure 9.3c). To attain a more isotropic resolution, the direction of the modulation has to be applied in several directions in the optical plane (Figure 9.3d).

An alternative, depictive approach to understand the resolution enhancing effect provided by SIM is to consider the Moiré effect, as shown in Figure 9.4. When two fine gratings are superposed, a third raw grating with a spatial period (or grating distance) $G_3$ occurs. If one of the fine original patterns with a spatial period $G_1$ is known and also the resulting pattern is known, while the second fine grating with $G_2$ is unknown, the unknown pattern can be reconstructed mathematically.

The known and the unknown fine grating represent the SIM excitation pattern and the high-frequency object information, respectively, whereas the raw grating represents the detected image.

9.2.2
Extracting the High-Resolution Information

When structured illumination is applied, the total spatial frequency information transmitted through the objective lens is increased. However, the additional information is overlaid with the original image information, as described in Section 9.2.1.
9.2 Structured Illumination Microscopy (SIM)

The additional information, therefore, has to be separated and shifted to the correct position in Fourier space. To separate the components, the acquisition of several images with different positions of the illumination pattern is required. By this method, the complex coefficients \((1, (m/2)e^{i\varphi}, \text{and } (m/2)e^{-i\varphi})\) of the image copies depending on the grating position \(\varphi\) (see Eq. (9.20)) can be calculated for the different grating positions consecutively. The image information belonging to the single copies can be separated afterward by the respective coefficient. When using two-beam interference, three images with different grating positions are sufficient to solve the equations; for three-beam interference, five images are necessary.

After separation, the copies of the image information can be shifted to their correct position by the reciprocal vectors of the illumination grating. When the correctly processed information of the different copies is added, the resulting image has a higher resolution than the original image.

As described above, the resolution improvement in the focal plane is anisotropic if the modulation of the illumination pattern is one dimensional in the lateral direction.

In order to achieve an almost isotropic resolution improvement nonetheless in this case, image series are taken at different angles of the periodic illumination pattern (typically at 0°, 60°, and 120° or at 0°, 45°, 90°, and 135°; Figure 9.3d).

It is also mathematically possible to use a two-dimensional grating (e.g., a hexagonal pattern) to generate the diffraction orders of the excitation light.

9.2.3 Optical Sectioning by SIM

Not only the lateral resolution as shown in the two-dimensional example in the paragraph above, is improved in SIM compared to standard wide-field microscopy...
but also the confocality is improved, resulting in an optical section with less signal from the out-of-focus region.

This effect becomes clear when the wide-field OTF (Figure 9.1) is considered. For the spatial frequencies $k_x, k_y = 0$, and $k_z \neq 0$, the OTF is always zero. A cone-shaped frequency region along the optical axis frequencies $k_z$ in the OTF is missing. The corresponding information is not transmitted by the microscope. This “missing cone” problem results in the fact that conventional microscopy suffers from a poor $z$-resolution.

As shown in Figure 9.5, the additional object information copies provided by SIM are capable of covering the missing cone of the original OTF and therefore yield an improved optical sectioning power of SIM compared to conventional fluorescence microscopy. However, in the case of two-beam illumination (Figures 9.2a,c, and 9.5a,c), confocality is improved only if the illumination pattern frequency is (slightly) smaller than the cut-off frequency. At the cut-off, the missing cones overlap and therefore remain uncovered.

As is the case with lateral resolution, it is difficult to quantify the axial resolution of a microscope and it is important to find a suitable model to calculate or measure comparable quantitative values for different microscopy methods. For example, the full width at half maximum (FWHM) of the 3D PSF in the axial dimension could be used. However, this $z$-direction FWHM depends on the $x$ and $y$ positions (Figure 2.12).

In the work of Karadaglic and Wilson (2008), the optical sectioning powers of two different SIM setups and of a confocal microscope were compared using a model of a thin fluorescent sheet located in the focal plane. The ability to determine the $z$-position of the sheet by the microscopes was compared.

It was shown that both two-beam interference (fringe projection) and three-beam interference (grid projection) SIM exhibit optical sectioning strengths comparable to that of a confocal microscope. However, three-beam interference provided better results in terms of confocality compared to two-beam interference. On the other hand, two-beam interference requires less single images at different phase

![Figure 9.5 Resulting OTF in two- (a,c) and three-beam illumination (b,d).](image-url)

The black spots in the microscope’s wide-field OTFs (a: two-beam and b: three-beam) show the origins of the shifted object information copies. When these copies are shifted back (c,d) to their correct positions, the effective OTF increases in size. The black outline shows the original wide-field OTF cut-off border. The borders of the shifted OTFs are shown in gray. The corresponding illumination patterns are shown in Figure 9.2a,b.
positions (3 instead of 5) for reconstruction, and because less object information copies are used, it is less susceptible to noise in the raw data.

The answer to the question whether two- or three-beam illumination is ultimately more favorable depends on the specimen that is analyzed.

9.2.4
How the Illumination Pattern is Generated

For structured illumination, a multitude of coherent light beams is necessary to generate the illumination conditions shown in Figure 9.2.

Basically, two techniques have been developed to arrange this: the application of a diffraction grating (Figure 9.6a) and the use of an interferometer (Figure 9.6b). In Section 9.2.6, examples for both ways are shown.

The two techniques differ in the dependence between the period of the illumination pattern and the wavelength of the excitation light (Figure 9.6).

In setups applying a diffraction grating in a conjugate image plane, there is an imaging condition: an image of the grating is projected into the object plane. Therefore, the spatial period of the illumination pattern is independent from the illumination light’s wavelength. Hence, the illumination light must not be coherent. An inherently incoherent light source (e.g., gas-discharge lamp or light-emitting diode (LED)) can be used instead of a laser.

For an interferometer setup, on the other hand, the illumination pattern has a period proportional to the wavelength of light. The larger the wavelength, the coarser the pattern becomes. Here, the light source has to be coherent in order to achieve a periodic intensity pattern (Figure 9.6b). For interferometer setups, the angle of the excitation light beams depends only on the angle of the interferometer. If this angle is set to a suitable value according to the objective lens’ numerical aperture (NA) once, a change in interference angle is not necessary for different wavelengths. For diffraction grating setups, a change in grating might be necessary if the wavelength is changed. As shown in Figure 9.6a, the red beams hit the objective lens at the utmost border, whereas the blue beams are close to the center of the objective lens.

9.2.5
Mathematical Derivation of the Interference Pattern

In the equations for the illumination pattern (Eq. (9.9)) and the derived statements, a variable modulation strength \( m \) was used.

To analyze where the modulation strength is originating from, the interference pattern for a two-beam interference SIM setup is derived. The two initial beams are considered to be equally strong and have equal linear polarization \( p_0 \). The right beam has a phase shift \( \varphi \) compared to the left beam.

In the following, the complex exponential form for the superposing waves is used instead of the trigonometric form. Both beams propagate identically along the \( z \)-direction before passing the objective lens. While passing the objective, the
Figure 9.6  Beam path and interference pattern for grating (a) and interferometer setup (b). In the interferometer setup shown here, the zero order of diffraction is blocked as well as 2 and \(-2\) orders and higher orders.

The left beam is rotated by the angle \(\alpha\) and the right beam by \(-\alpha\) (Figure 9.7) around the \(y\)-axis.

Before passing the lens, the electric fields of the left and the right beam are given by

\[
E_{l0} = p_0 E_0 e^{i(k_0 r - \omega t)}
\]  

(9.22)

and

\[
E_{r0} = p_0 E_0 e^{i(k_0 r - \omega t - \phi)}
\]  

(9.23)
For the original wave vector \( \mathbf{k}_0 \) points along the \( z \)-direction:

\[
\mathbf{k}_0 = \begin{pmatrix} 0 \\ 0 \\ \frac{2\pi}{\lambda} \end{pmatrix}
\] (9.24)

After the lens, the rotated polarizations of the beams are given by

\[
\mathbf{p}_l = R_y(\alpha) \mathbf{p}_0 = \begin{pmatrix} \cos \alpha & 0 & \sin \alpha \\ 0 & 1 & 0 \\ -\sin \alpha & 0 & \cos \alpha \end{pmatrix} \mathbf{p}_0
\] (9.25)

and

\[
\mathbf{p}_r = R_y(-\alpha) \mathbf{p}_0 = \begin{pmatrix} \cos \alpha & 0 & -\sin \alpha \\ 0 & 1 & 0 \\ \sin \alpha & 0 & \cos \alpha \end{pmatrix} \mathbf{p}_0
\] (9.26)

through application of the rotation matrix \( R_y \) around the \( y \)-axis.

The same rotation accounts for the wave vectors, which yields

\[
\mathbf{k}_l = \begin{pmatrix} \sin \alpha \\ 0 \\ \frac{2\pi}{\lambda} \cos \alpha \end{pmatrix}
\] (9.27)

\[
\mathbf{k}_r = \begin{pmatrix} -\sin \alpha \\ 0 \\ \frac{2\pi}{\lambda} \cos \alpha \end{pmatrix}
\] (9.28)

\[
\mathbf{E}_l = \mathbf{p}_l \mathbf{E}_0 e^{i(k_l r - \omega t)}
\] (9.29)

\[
\mathbf{E}_r = \mathbf{p}_r \mathbf{E}_0 e^{i(k_r r - \omega t - \phi)}
\] (9.30)

The superposition \( \mathbf{E}_s = \mathbf{E}_l + \mathbf{E}_r \) in the object plane yields

\[
\mathbf{E}_s = \mathbf{E}_0 \left( \mathbf{p}_l e^{i(k_l r - \omega t)} + \mathbf{p}_r e^{i(k_r r - \omega t - \phi)} \right)
\] (9.31)

Figure 9.7  Polarization of the beams before and after passing the objective lens for polarization perpendicular (s; (a)) and parallel (p; (b)) to the interferometer plane.
The intensity is proportional to the absolute square of the electric field:

\[ I \propto |E_0|^2 = a |E_s|^2 = a E_s E_s^* \]  

(9.32)

with a proportionality constant \( a \). By substituting Eq. (9.31) in Eq. (9.32), the intensity becomes

\[ I = 2a E_0^2 \left( 1 + p_l p_r \cos \left( (k_l - k_r) r + \phi \right) \right) \]  

(9.33)

after some rearrangements. Substituting the wave vectors \( k_l \) and \( k_r \) (Eqs (9.27) and Eq. (9.28)) yields

\[ I = 2a E_0^2 \left( 1 + p_l p_r \cos \left( \frac{4\pi \sin \alpha}{\lambda} x + \varphi \right) \right) \]  

(9.34)

Obviously with \( p_l p_r = m \) and \( 2a E_0^2 = I_0 \), the term is identical to the illumination pattern of a two-beam interference SIM microscope (Eq. (9.9)).

When both interfering beams of a two-beam interference SIM setup are equally strong, the modulation strength depends on the primary polarization of the beams and on the angle \( \alpha \) by which the beams are deflected by the objective. To examine these effects, we compare two extreme cases of linear polarization: polarization perpendicular (s)

\[ p_{0s} = \begin{pmatrix} 0 \\ 1 \\ 0 \end{pmatrix} \]  

to the interferometer plane and parallel

\[ p_{0p} = \begin{pmatrix} 1 \\ 0 \\ 0 \end{pmatrix} \]  

to the interferometer plane (Figure 9.7). The interferometer plane is the plane that is spanned by the two beams.

If the polarization of the incoming beams is perpendicular (s), the polarization of the single beams (\( p_l, p_r \)) will not be changed by passing through the objective lens (application of \( R_y(\alpha) \) and \( R_y(-\alpha) \)). For beams parallel (p) to the interferometer plane, owing to the change in beam angle, the polarization of the left beam is rotated by \( \alpha \) and of the right by \( -\alpha \). For \( p \) polarization and \( \alpha = 45^\circ \), the polarizations and therefore the electric fields of both beams are perpendicular. The modulation strength \( m = p_l p_r \) becomes zero and the resulting intensity of the object plane becomes constant (Figure 9.8b). Usually, the interferometer plane is rotated to different angles (usually 0°, 60°, and 120°) around the optical axis in the SIM image acquisition process. To achieve a high modulation strength for all angles, the polarization of the excitation light has to be rotated with the pattern to allow for s-polarization in every case. This can be done, for example, with a rotatable half-wave plate or an electro-optic modulator.

### 9.2.6 Examples for SIM Setups

In SIM, the excitation intensity in the focal plane is a periodic pattern. To achieve this illumination distribution, various different setups have been established. Commonly, the excitation light is projected through the same objective lens that
is used for the detection of the fluorescence in order to generate a standing wave field, even though other illumination techniques with the use of additional optical devices or even near field excitation are possible.

Most commonly, SIM microscopes use either a physical grating (Heintzmann and Cremer, 1999; Gustafsson, 2000) or a synthetic grating generated by a spatial light modulator (SLM) (Hirvonen et al., 2008; Kner et al., 2009) in an intermediate image plane to create the modulated pattern in the object plane (Figure 9.9).

When a grating is used, usually the setup is applied in a way that only the zero- and the first-order diffractions of the excitation beams enter the objective lens. The use of higher orders (five and more beam interference) is possible but would lead to a more complicated separation of the copies of the Fourier-transformed object information and a lower signal-to-noise-ratio as the number of copies would be higher. The zero-order diffraction (undiffracted beam) can optionally be blocked in order to apply two-beam interference; the use of zero, plus, and minus first order leads to the case of three-beam interference.

The phase (i.e., position) of the interference pattern in the object plane can be shifted by moving the diffraction grating in the intermediate image plane. To change the orientation of the pattern’s modulation, the grating can be rotated.

Alternatively, a two-dimensional grating can be applied to generate a pattern with modulation along multiple axis in the object plane, as noted in the previous section.
When an SLM is used, the pattern can be shifted and rotated by displaying different gratings on the SLM. Scientific SLMs usually consist of an array of liquid crystals to modify polarization and/or phase of light individually for the separate pixels (a conventional liquid crystal display (LCD) is an SLM, too).

A fast SLM exhibits speed advantages over a solid grating and is also more versatile because the grating’s lattice spacing can easily be adjusted to the used wavelength. On the other hand, a solid physical grating has certain advantages over the use of an SLM. There is less intensity lost and the phase of the intensity light is not altered spatially when passing the grating, which leads to a stronger modulation of the intensity pattern in the object plane.

A different approach is to use an interferometric setup to generate the coherent beams and channel them toward the objective. A particular setup applying an interferometer (Best et al., 2011) (Figure 9.10) is based on a Twyman–Green interferometer, a special case of a Michelson interferometer applying a collimated, widened beam. The used interferometer consists of a beam splitting cube and two opposing mirrors.

This setup is based on an inversely applied specially designed microscope. The excitation laser beam is directed to a 50% beam splitting cube (Figure 9.10, cube...
A), positioned in a focal point of the focusing lens. Half of the beam is reflected by 90° as the other half passes through the cube without a change in direction. The resulting two beams are then reflected with mirrors by 180° back into the cube. After the light passes the beam splitter, again, two beams, each at 1/4 intensity, are generated, which leave the cube channeled toward the focusing lens. If the beam splitting cube is rotated around the axis perpendicular to the table by an angle $\theta$, one beam is deflected by $2\theta$, as the other beam is deflected by $-2\theta$. Thus, the interference pattern can be adjusted by rotating the beam splitter. The beams pass the focusing lens and are then deflected by a dichromatic beam splitter toward the objective. After passing focusing lens and objective, the interference of the two beams in the object plane leads to a sinusoidal pattern with modulation parallel to the object plane. The orientation of the modulation can be changed by rotating the beam splitter around an axis parallel to the ground and perpendicular to the previous rotation axis. It is therefore possible to generate sinusoidal interference with arbitrary period and direction, which makes it possible to adjust the period of the pattern in accordance to the particular task and wavelength. A webcam can be used to monitor the interference pattern directly. The microscope also offers the option to use a third beam, not deflected by an angle and positioned central to the two outer deflected beams, in order to generate a three-beam interference pattern.
pattern. The beam splitter extracting the central beam (C) is located in the beam line previous to the other splitters. The phase of the pattern can be altered by shifting the relative phases of the separate beams. To accomplish this accurately and quickly, piezo actuators are attached to the mirrors facing the rotatable beam splitting cube (A).

9.3 Spatially Modulated Illumination (SMI) Microscopy

9.3.1 Overview

SMI is a microscopy method that can be used for precise size measurements of small fluorescent objects.

In SIM, the excitation light comes from one objective lens and the interference pattern is spanned along the focal plane (e.g., \(x - y\)-plane) whereas in SMI microscopy, two counterpropagating waves from two opposing objective lenses are used (Figure 9.11). The specimen is located between the two objectives, where the two beams form a standing wave pattern. Here, the interference pattern modulates only along the optical axis (\(z\)-direction).

Because the two beams are counterpropagating, the period of the interference pattern fringe distance \(d\) is very small (i.e., it has a high frequency). Analog to the case in SIM, the relation that the finer the pattern, the higher the resolution is valid. When the resulting OTF is considered (Figure 9.12b), as in Figure 9.3 and Figure 9.5 for SIM, in the SMI case, copies of the OTF appear shifted far in the \(z\)-direction of spatial frequencies \(k_z\). The OTF copies have no overlap with the wide-field OTF in the center, which means that the supported frequency region of the resulting OTF is not continuously interconnected. Even though information

![Figure 9.11 SMI setup. Two coherent light beams propagate through two opposing objectives and interfere in to object plane with a fringe distance \(d\).](image_url)
of very high-resolution frequencies in the axial direction (dashed structures in Figure 9.12b) is transmitted by the microscope, information from large frequency regions in between is not accessible.

As a consequence, the generation of high-resolution images free of artifacts as done by SIM is not possible by this method because large fractions of the object’s moderately high-resolution information get lost.

Therefore, *a priori* knowledge concerning the analyzed specimen is necessary in order to use SMI microscopy. However size and position measurements of small objects can be done with a precision far beyond the diffraction limit (typically 40 nm) with this method.

### 9.3.2 SMI Setup

A typical SMI setup is shown in Figure 9.13. In order to generate two coherent and collimated beams, a collimated laser beam is split using a beam splitter. Each beam passes an additional lens for each objective, where the focal plane of the lens is located in the back focal plane of the objective. This converts the objective and the focusing lens into a collimator. With this optical setup, an interference pattern of sinusoidal shape along the optical axis can be generated between the objective lenses. The sample is placed between the objective lenses and can be moved along the optical axis through the interference pattern. The detection, which is equal to conventional wide-field fluorescence detection, is realized by only one objective lens, which is used in conjunction with a dichroic mirror to separate the excitation light from the fluorescence signal. During image acquisition the object is moved in precise axial steps (e.g., each 20 or 30 nm) through the standing wave field. At each step, a fluorescence image is registered.
Figure 9.13  Horizontal SMI setup with three different laser sources, one LED for common transmitted light illumination and one monochrome charge-coupled device (CCD) camera. (Source: Reprinted from Reymann, 2008, with kind permission from Springer Science+Business Media.)

9.3.3 The Optical Path

The fringe period of the standing wave located in the object plane depends on the excitation wavelength $\lambda$, the refractive index $n$ of sample, and slide, as well as a possible tilting angle $\theta$ of the beams relative to the optical axis (Figure 9.11).

In the following the mathematical expression for the intensity distribution is derived.

Two counterpropagating coherent electromagnetic waves $E_i$ and $E_r$ interfere in the focal region. It is assumed that both beams have the same amplitude $A$ and that both beams might be rotated around the y-axis by an angle $\vartheta$. The beam passing through the right objective (Figure 9.13) has a phase delay relative to the left beam of $\varphi$. The result is a periodic standing wave field $E_s$ with an intensity distribution $I_s$.

The calculation of the intensity distribution of the SMI interference pattern can be derived from the equation for the pattern of a two-beam interference
SIM (Eq. (9.33)):

\[ I = I_0 \left( 1 + p_l p_r \cos \left[ (k_l - k_r) r + \varphi \right]\right) \]  

(9.35)

In the SMI case (Figure 9.11), the wave vectors of the two beams are given by

\[ k_l = \begin{pmatrix} \sin \theta \\ 0 \\ \cos \theta \end{pmatrix} k \]  

(9.36)

\[ k_r = \begin{pmatrix} \sin \theta \\ 0 \\ -\cos \theta \end{pmatrix} k \]  

(9.37)

with the absolute value \( k \) of the wave vector \( k \).

\[ k = |k| = \frac{2 \pi}{\lambda} \]  

(9.38)

The polarization is considered to be parallel to the \( y \)-axis (\( s \)-polarization):

\[ p_l = p_r = \begin{pmatrix} 0 \\ 1 \\ 0 \end{pmatrix} \]  

(9.39)

The intensity becomes

\[ I = I_0 \left( 1 + \cos [2 \cos \theta kz + \varphi] \right) \]  

(9.40)

The illumination pattern can be phase shifted by changing the optical path length \( \Phi \) of the interferometer. \( k \), the norm of the wave vector \( k \), is expanded by the refractive index factor \( n \) of the media as the wavelength of light is inversely proportional to \( n \):

\[ \lambda = \frac{\lambda_0}{n} \]  

(9.41)

\[ \Rightarrow k = k_0 \; n; \quad k_0 = \frac{2 \pi}{\lambda_0} \]  

(9.42)

The grating distance \( G_{SMI} \) of the pattern in \( z \)-direction can be derived from Eq. (9.35) by

\[ (k_l - k_r) = \begin{pmatrix} 0 \\ 0 \\ G_{SMI} \end{pmatrix} = 2kG_{SMI} \cos \theta = 2\pi \]  

(9.43)

\[ G_{SMI} = \frac{\lambda_0}{2n \cos \theta} \]  

(9.44)

This means that for samples with a thickness larger than \( G_{SMI} \), multiple interference fringes are located inside this volume and as a result, no unique phase position might be distinguishable. Several maxima of the illumination pattern might be located in the depth of the sample at once independently of the actual phase of the pattern.

For a well-aligned system with a tilting angle below 10° (\( \cos \theta \approx 1 \)), the cosine term in Eq. (9.44) can be neglected. A fringe period is obtained that is proportional
to the vacuum wavelength $\lambda_0$ divided by two times the refractive index. Using immersion media with high refractive index, $n$ will be roughly 1.5. In this case, the fringe distance is $\lambda$ divided by 3. Therefore, an excitation wavelength of 488 nm results in a fringe distance of approximately 163 nm. This means that the $\cos^2$-intensity profile of the excitation has a maximum every 163 nm. When this value is compared with the axial depth of focus ($\sim$600 nm for an NA 1.4 objective lens), it can be seen that there is more than one intensity maximum within the focal depth (Figure 9.12a). Hence, it is possible to achieve information from the high-resolution region beyond the classical resolution limit. The approach is described in the following sections.

9.3.4
Size Estimation with SMI Microscopy

There are two major effects, which occur when moving the sample through the focus of the detection objective lens. On the one hand, the detected intensity is modulated by the interference of the excitation pattern, while the modulation contrast depends on the object size. On the other hand, there is a variation of intensity between objects in the focus and out of the focus.

The analysis of SMI data is primarily based on these two effects. The axial PSF of a wide-field microscope is given by a $\sin^2$ function (Chapter 2). As a result of the two-beam interference, the excitation intensity distribution is given by a $\cos^2$ function.

When the illumination pattern is kept fixed to the object plane and a pointlike object is moved along the $z$-direction, two effects superpose. The illumination intensity will vary sinusoidally because of the illumination pattern and the image of the object will move through the focus. As a result, the illumination pattern and the wide-field PSF are multiplied to form the resulting SMI-PSF (Figure 9.14). Figure 9.12 shows the 3D SMI-PSF on the left and the corresponding OTF on the right.

The axial SMI-PSF shows as envelope the $\sin^2$ function, which is modulated by a $\cos^2$ interference pattern. In this example of an infinitely small pointlike object,

![Figure 9.14](image)

Figure 9.14 Axial detection PSF (left), excitation pattern (middle), and resulting SMI point spread function (right).
the modulation contrast $R$ is 1. The modulation contrast $R$ is defined as follows:

$$R = \frac{I_{\text{max}} - I_{\text{min}}}{I_{\text{max}}}$$  \hspace{1cm} (9.45)

where $I_{\text{max}}$ and $I_{\text{min}}$ represent the maximum and minimum intensity values of the inner and outer SMI-PSF envelopes, respectively. In other words, the detected intensity light modulation and the envelope function from the axial detection of the objective lens (Schneider et al., 1999; see also Figure 9.14):

$$\text{AID} = \left( \frac{\sin \left( k_1 z \right)}{k_1 z} \right)^2 \times \left( a_1 \times \cos^2 \left( k_2 z \right) + a_2 \right)$$  \hspace{1cm} (9.46)

In this equation, $k_1$ represents the FWHM of the envelope function, $k_2$ is the wave vector of the standing wave field, $a_1$ represents the modulation strength, and $a_2$ defines the modulation contrast. For simplification, additional phase offset values and nonmodulated parameters are neglected in this formula.

When, instead of a hypothetical point-shaped object, a large object is analyzed, the modulation contrast is smaller than 1 and varies with the object’s size and geometry (Failla et al., 2002; Albrecht et al., 2001; Wagner, Spöri, and Cremer, 2005). When analyzing biological nanostructures, it is often suitable to assume a spherical geometry. Figure 9.15 shows the modulation contrast $R$ in dependence of the diameter of a spherical object. Obviously the modulation contrast graph is not bijective. For a given modulation contrast, there might be several solutions for the size of the object. The modulation contrast for spherical objects is 0 for certain object sizes (240 and 415 nm for 488 nm excitation wavelength). This means that the emitted intensity does not vary when the illumination pattern is shifted through the specimen for these object sizes.

If the application of SMI is constrained to the case of a modulation contrast beyond 0.18, which corresponds to an object size of roughly 200 nm, the relation is bijective and therefore can be used for size determinations. Above this size limit, conventional microscopy can be used to determine the object’s size. The

![Figure 9.15](image)
smallest diameter is limited by the shape of the modulation contrast function and the amount of detected photons. Typically, sizes below 40 nm can be measured by SMI with high precision.

The graphs in Figure 9.15 were calculated for the case of a spherical object with a homogeneous fluorophore density distribution (Wagner et al., 2006). If the object is small compared to the interference pattern, the detected fluorescence signal is similar to Figure 9.16a.

When the diameter of the object is increased (Figure 9.16b), the modulation depth is decreased, but the fringe distance stays the same and the envelope function also roughly stays the same. As an extreme case, the modulation contrast $R$ will disappear when the object diameter becomes significantly larger than the fringe distance. By the use of the simulated modulation contrast graph (Figure 9.15), it is possible to extract a value for the diameter of a diffraction limited object from the measured modulation contrast. It is important to keep in mind that this value is a calculated value under some assumptions – especially the geometry of the object has to be defined a priori – and that this value may differ from the “true” size if the assumptions are incorrect.

9.4 Application of Patterned Techniques

Patterned techniques have become an important tool in microscopic analysis of biomedical subjects. Today, every major microscopy manufacturer features a SIM system.

In this paragraph, some brief examples for applications of patterned techniques are discussed.

One of many applications of SIM is the analysis of autofluorescent structures in tissue of the eyeground. Degradation of the retinal pigment epithelium (RPE) is responsible for the age-related macular degeneration (AMD), the main reason for blindness in the developed world in the elderly population. This disease, where vision in the macular (the region of sharpest sight) is progressively lost, is linked to excessive aggregations of autofluorescent compound in RPE cells. The RPE is
a cell monolayer between the retina and the choroid. It has essential functions in sustaining the vision process. The compound called lipofuscin accumulating in the RPE cells is autofluorescent, which makes it easily detectable by fluorescence imaging without the need for additional labeling.

The images presented here have been generated with an interferometric SIM setup (Best et al., 2011). Paraffin sections of human retinal tissue have been cut, deparaffined, and prepared on object slides (Ach et al., 2010). The specimens were analyzed using three different excitation wavelengths (488, 568, and 647 nm). The use of different excitation wavelengths showed a spatially varying composition of the fluorescent granules.

When comparing the acquired SIM images with the standard wide-field data, a considerable improvement is apparent (Figures 9.17 and 9.18). Not only the contrast is greatly improved, but also the optical resolution is enhanced by a factor of 1.6–1.7 depending on the wavelength. Thus, previously irresolvable details can be

![Figure 9.17](image1.png)
![Figure 9.17](image2.png)

**Figure 9.17** Multicolor SIM image of retinal pigment epithelium (RPE). The colors red, green, and blue represent the signal for 647, 568, and 488 nm excitation, respectively. The SIM image (b) shows more details and less out-of-focus light than the wide-field image (a). The background of each channel is subtracted and each channel is stretched to full dynamic range. The Bruch membrane (B) is located between the choroid (C) and the retinal pigment epithelium (RPE). On the top of the image, endings of retinal rod cells (R) can be seen. The scale bar is 2 µm (Best et al., 2011).
discovered. Figure 9.18 shows the intensity along a line through structures in RPE cells below 488 nm excitation each in a wide-field image and the corresponding structured illumination image. The gray value (intensity) of both images was normalized to full 8-bit range and the background intensity was subtracted.

The improvement of contrast in the SIM image is apparent by the efficient suppression of out-of-focus light, resulting in higher dynamic in the examined region.

The lateral resolution enhancement allows additional details to be seen (e.g., the two circular structures in the center of the image can clearly be separated in the SIM image). The suppression of out-of-focus light allows for the generation of 3D images. SIM images at several axial focal positions are acquired and the resulting high-resolution data is combined into a 3D data set. The acquisition speed of SIM is sufficient to allow super-resolution living cell imaging, as shown in Figure 9.19.

The other microscopy technique treated in this chapter, the more specialized method SMI, is suited to analyze the size of small nanostructures in biological specimens.
Figure 9.19 3D-SIM image of autofluorescent lipofuscin granules inside of a living RPE cell.

Figure 9.20 SMI analysis of DNA replication foci. (b) Shows thresholded foci from the raw data (a). (c) Shows the size of the foci (the black ones had to be ignored because of insufficient signal level or a size outside of the effective range). (d) Shows the axial position of the analyzed foci (Baddeley et al., 2010). © 2010, Oxford University Press.
Baddeley et al. (2010) showed that SMI can be used to measure the size of DNA replication foci in the nucleus of mammalian cells. For this study, both SMI and SIM have been used.

Both methods showed an average size of 125 nm independent of the labeling method. Remarkably, the super-resolution methods were able to resolve three- to fivefold more distinct replication foci than previously reported. For the SMI data analysis, the data stack was first filtered for pointlike objects. For the identified objects, an axial profile was extracted that was then analyzed with the SMI methods described in Section 9.3.4 to determine the accurate size. A spherical geometry was assumed for the single replication foci (Figure 9.20).

9.5 Conclusion

Interference techniques deliver increased optical resolution. The main benefit of these techniques compared to other super-resolution techniques is that no special requirements for the applied fluorochromes are necessary. No photoswitching or saturation effects (nonlinear effects) are used to attain the increase in resolution because it is based solely on a change in the intensity distribution of the excitation light compared to standard fluorescence microscopy. However, super-resolution methods based on nonlinear effects have the intrinsic advantage of a higher resolution than SIM (theoretically unlimited).

Still, the ease of use makes SIM the high-resolution method of choice for many biomedical applications. In contrast to most other high-resolution techniques, SIM allows living cell analysis because of relatively short acquisition times and low excitation intensities. Compared to conventional confocal microscopy, SIM delivers a superior image quality and optical resolution at a comparable applicability. Even though linear patterned techniques cannot compete directly with localization methods for single molecules (Chapter 8) in terms of positioning accuracy, they can deliver complementary information. When both methods are combined, high structural resolution, on the one hand, and high localization accuracy, on the other hand, can be realized.

9.6 Summary

Fluorescence microscopy techniques using patterned illumination light offer the opportunity to extract high-resolution object information beyond the conventional resolution limit. Especially the SIM technique, where laterally modulated illumination through one objective lens is used, has evolved to become an important tool for high-resolution imaging in biomedical research. Because of the interplay of the illumination pattern with the object’s spatial frequencies, in SIM a resolution
improvement by a factor of 2 is possible. In this chapter, a fundamental mathematical description of this method is provided and different variations of SIM setups are described. The secondary focus of this chapter lies on the less common method of SMI where two opposing objective lenses are used to generate a high-frequency interference pattern along the optical axis. The SMI method is used to measure the size of nanostructures in great precision. At the end of the chapter, a brief description of exemplary biological applications for the two patterned illumination methods is provided.

Acknowledgments

We thank Stefan Dithmar and Thomas Ach from the University Hospital Heidelberg for their kind cooperation and funding. We thank Rainer Heintzmann for his support and for providing his reconstruction software for structured illumination microscopy. We gratefully appreciate the support of the members of the group of Christoph Cremer, especially the help of Margund Bach and Udo Birk.

References


