Combination of tissue optical clearing and 3D fluorescence microscopy for high-throughput imaging of entire organs and organisms

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CONTENTS
Introduction ..................................................................................................................................................................................277
Principle of traditional TPEM ..................................................................................................................................................277
Limitations of traditional TPEM application by light scattering .................................................................................................279
Tissue optical clearing with TPEM ..............................................................................................................................................281
Tissue optical clearing with LSFM ..............................................................................................................................................281
Principle of conventional LSFM ..................................................................................................................................................283
Three challenges and corresponding methods for LSFM ............................................................................................................284
Conclusions and outlook ..............................................................................................................................................................292
Acknowledgments ........................................................................................................................................................................292
References ..................................................................................................................................................................................293

Introduction

Biologists have always wanted to observe various organs and tissues to study the structure, function, and dysfunction of their cell components. In the past, this often required tissue extraction and histological preparation to gain access. Traditional optical microscopy techniques use a linear (single-photon) absorption process to produce contrast, but they are limited to high-resolution imaging near the tissue surface (less than 100 µm) because of the strong and multiple light scattering at greater depths. Scattering particularly severely affects the signal intensity in confocal microscopes. Confocal microscopes can achieve high three-dimensional resolution and optical sectioning by pinholes which reject all light that does not appear to come from the focus.

In the past two decades, a new optical microscope technology has been developed that uses nonlinear light-mass interactions to generate signal contrast. Nonlinear optical microscopy technology has a special function that reduces its sensitivity to scattering, so it is very suitable for high-resolution imaging in tissues. In particular, the two-photon excited fluorescence laser scanning microscope (2PLSM), combined with in vivo fluorescence labeling technology, has opened up a rapidly expanding field for imaging research of intact tissues and living animals.

Although 2PLSM is currently a mature technology that uses turn-key laser sources and commercial microscope systems, it is still important to understand its basic principles and key technologies, especially when optimizing the microscope system to achieve a large imaging depth. In this review, we discuss the physical principles, especially focusing on the imaging system parameters that are important for deep imaging, and summarize the technical issues related to the application of 2PLSM in high-resolution imaging of live animals.

Principle of traditional TPEM

In optical microscopy, one can distinguish between linear and nonlinear excitation. Traditional techniques, including confocal microscopy, generate contrast from light–matter interactions, in which the elementary process involves a single photon and which therefore depend linearly on the incident light intensity. Nonlinear techniques are fundamentally different in that they use “higher-order” light–matter interactions involving multiple photons for contrast generation. The nonlinear nature of these interactions leads to qualitatively new imaging properties.

Several different nonlinear processes can occur when light interacts with matter (Figure 14.1). Most widely used in biological imaging is fluorescence excitation by two-photon absorption [1]. Two photons that arrive “simultaneously” (within ~0.5 fs) at a molecule combine their energies to promote the molecule to an excited state, which then proceeds along the normal fluorescence-emission (or photochemical-reaction) pathway [1, 2]. Similarly, three or more photons can combine to cause excitation.

The efficiency of multiphoton absorption depends on the physical properties of the molecule (the “multiphoton absorption cross-section”) [3, 4], and on the spatial and temporal...
Combination of tissue optical clearing distribution of the excitation light. Most nonlinear processes have in common that the transition probabilities are extremely low at “normal” light intensities. To generate sufficient signal, excitation light has to be concentrated in space and time. High spatial densities are (cheaply) generated by focusing a laser beam through a high numerical aperture (NA) objective. Concentration in the time domain requires the use of (expensive) lasers that emit “ultrashort” pulses (less than a picosecond long) with correspondingly high peak intensities. For laser pulses of width \( \tau \) occurring at a rate \( f_R \), the signal is enhanced by a factor of \( 1/(\tau f_R)^{n-1} \) compared to continuous-wave illumination, where \( n \) is the number of photons involved in the elementary process. Lasers typically used in TPEM provide 100-fs pulses at about 100 MHz, with a “two-photon advantage” of about 10 [5].

Multiphoton absorption is but one of several possible nonlinear interactions [5]. Another is optical-harmonic generation, in which two or more photons are “simultaneously” scattered, generating a single photon of exactly twice (thrice, and so on) the incoming quantum energy (Figure 14.1a). Harmonic generation requires no actual absorption but is enhanced near a resonance, albeit at the expense of parasitic absorption [6]. It also differs from multiphoton absorption in that it is a coherent – that is, phase-preserving – process, which causes speckles, possible cancellation, predominantly forward-directed emission, and supralinear dependence on the chromophore density. In practice, only second-harmonic [6] and third-harmonic [7, 8] generation have been used. Second-harmonic (but not third-harmonic) generation depends on the absence of inversion symmetry, which not only requires that individual molecules are inversion-asymmetric (as most biological molecules are) but also that they are spatially ordered. Second-harmonic generation has, therefore, been useful for investigating ordered structural protein assemblies such as collagen fibers [9] or microtubules [10]. Similarly, dyes that are incorporated preferentially in one leaflet of the plasma membrane can be used to detect membrane voltage [11, 12]. A further process used for microscopy is coherent anti-Stokes Raman scattering (Figure 14.1a), which is sensitive to molecular vibration states and can be used to detect the presence of specific chemical bond types [13, 14].

All nonlinear microscopy techniques require expensive pulsed laser systems to achieve sufficient excitation rates. Two major advantages make the investment worthwhile. First, because multiple excitation photons combine their quantum energies in nonlinear microscopy, the photons generated (or the transitions excited) have higher energies than the excitation light, making emission “bluer” than the excitation, which
is different from traditional fluorescence. For commonly used fluorescent markers, multiphoton absorption occurs in the near-infrared wavelength range (700–1000 nm), whereas emission occurs in the visible spectral range. Near-infrared light not only penetrates deeper into scattering tissue but is also generally less phototoxic owing to the lack of significant endogenous (one-photon) absorbers in most tissues.

The second major advantage of two-photon absorption and, in fact, of all nonlinear contrast mechanisms, is that the signal (S) depends supralinearly ($S \propto I^n$) on the density of photons, that is, the light intensity (I). As a consequence, when focusing the laser beam through a microscope objective, multiphoton absorption is spatially confined to the perifocal region (Figure 14.1b). The absence of multiphoton absorption in out-of-focus planes contrasts with confocal microscopy, where (single-photon) absorption occurs within the entire excitation light cone. The lack of out-of-focus excitation in nonlinear microscopy further reduces photodamage and thus increases tissue viability, which is crucial for long-term imaging [16]. Localization of excitation also provides excitation-based three-dimensional resolution with no need for spatially resolved detection through a confocal pinhole. By the same token, multiphoton absorption allows highly localized photon manipulations, such as photobleaching and photolytic release of caged compounds, within femtoliter volumes [17–19].

Localization of excitation is maintained even in strongly scattering tissue because the density of scattered excitation photons generally is too low to generate a significant signal, making nonlinear microscopy far less sensitive to light scattering than traditional microscopy (Figure 14.2). This is of paramount importance for deep imaging, because it means that all fluorescence photons are known to originate from near the focus and thus can provide useful signal. However, the deep imaging described above often only involves samples at the micron level, and imaging depths of 1 mm are currently extremely difficult to achieve and need to be combined with other advanced imaging techniques. These additional add-ons raise the cost of the device while also placing higher demands on the construction of the optical path. In the following subsection, the implications and limitations of conventional two-photon excitation microscopy due to the effects of sample scattering are detailed.

### Limitations of traditional TPEM application by light scattering

In most biological tissues, absorption of light is negligible compared to scattering, particularly in the near-infrared wavelength range. Scattering is the deflection of a light “ray” from its original direction; if the photon energy stays unchanged, it is termed elastic. Elastic scattering depends on refractive index inhomogeneities, which are present even in glass but are much stronger in tissue because cells are a heterogeneous mixture of molecules and supramolecular structures with varying molecular polarizabilities. The strength of scattering is described by the mean free path ($l_t$), the average distance between scattering events. The likelihood and angular distribution of scattering depend on refractive index variation, object size, and wavelength $\lambda$. For very small objects (such as isolated atoms or molecules in a gas) scattering is nearly isotropic and strongly wavelength-dependent ($\propto \lambda^{-4}$; Rayleigh scattering). For objects comparable in size to the wavelength (as in cells), scattering is directed mostly in the forward direction. This can be quantified by the anisotropy parameter ($g$) or by the transport mean free path, $l_t = l_s / (1 - g)$, which is the distance after which “direction memory” is lost. Measurements in brain gray matter yielded values for $l_s$ of 50–100 $\mu$m at 630 nm in extracted tissue and of about 200 $\mu$m at 800 nm in vivo [20, 21]. Scattering decreases with wavelength, albeit less than expected for Rayleigh scattering [21]. The anisotropy parameter generally is high ($\approx 0.9$) in brain tissue [22]. In nonlinear optical microscopy, only ballistic (nonscattered) photons contribute to signal generation in the focal volume. The ballistic power follows a Lambert-Beer–like exponential decline with imaging depth $z$

$$P_{\text{ball}} = P_0 e^{-zl_t}$$

(14.1)

with length constant $l_t$ and surface power $P_0$. Because of the quadratic intensity-dependence in TPEM, the fluorescence signal declines as

$$F_{\text{2PE}} \propto \left(e^{-zl_t}\right)^2 = e^{-2zl_t}$$

(14.2)
Conversely, we need an exponentially increasing laser power entering the surface \( (P_0 \propto e^{-g}) \) to maintain the same ballistic intensity at the focus. The reduction in focal intensity depends on \( L \) and not \( l \), because even a small deflection from the original path causes a ray to miss the focus (Figure 14.2). In contrast, the forward-directed angular distribution of scattering is important for the calculation of the near-surface background. Both \( l \) and \( g \) not only depend on tissue type but can also change substantially with age and upon removal of the tissue from the animal [21]. Surface scattering can become important if the beam crosses between media with substantially different refractive indices, for example when imaging through the skull. Scattering of fluorescence photons is important for the detection process. Because of the short mean free path of visible light, the ballistic fraction becomes quickly irrelevant with increasing focal depth. For sufficient depth, multiply scattered fluorescence light leaves the sample from a diffusely radiating region on the surface, which has a full-width-at-half-maximum intensity (FWHM) of about \( 1.5 \times \) the focal depth, independent of the scattering length [23].

The maximum achievable imaging depth is proportional to the scattering-mean-free-path and depends logarithmically on available laser power, two-photon advantage, and collection efficiency [21]. With a laser oscillator providing \( \sim\)100-fs pulses, the maximum depth usually is limited by the available average power (1 W average power allows imaging depths of about 600–800 \( \mu \)m in the neocortex) [20, 24–27]. When a regenerative amplifier is used, it is possible to image deeper (up to 1 mm in the neocortex) [28]. Eventually, however, fluorescence generated near the sample surface becomes a limiting factor. The resulting contrast reduction might be impossible to overcome in samples with a wide fluorophore distribution as, for example, in transgenic mice with extensive GFP expression. The achievable imaging depth also strongly depends on other tissue properties such as microvasculature organization, cell body arrangement, and collagen or myelin content, which will more or less degrade the laser focus and limit signal generation deep inside the tissue. If deeper structures need to be reached and if less-scattered longer wavelengths cannot be used, mechanical penetration or removal [29] of overlying tissue may be necessary. Efforts in this direction have been made using very narrow objective lenses made from gradient-index (GRIN) material [30–32].

In clear tissue, all excitation light reaches the focus, but in scattering tissue, scattering (even by a small angle) causes light rays to miss the focus and be lost to signal generation. This leads to a roughly exponential decrease in excitation with depth. In clear tissue only fluorescence light rays initially emitted into the collection cone, determined by the objective’s NA, can be detected, but in scattering tissue, fluorescence light is (multiply) scattered and may even “turn around.” Fluorescence light apparently originates from a large field of view but a larger fraction than in the nonscattering case is actually within the angular acceptance range \( \theta_l \) of the objective.

Besides normal imaging of brain slices, TPEM is also used for high-resolution imaging in various organs of living animals. This chapter shows additional aspects that are important for \textit{in vivo} imaging, using mainly examples in the intact brain.

For short-term studies of the brain, a cranial window is opened above the area of interest, which also provides access for recording electrodes (Figure 14.3a). Other organs are surgically exposed in a similar manner or, as in the case of kidney, are exteriorized [33]. A general problem for \textit{in vivo} imaging is motion induced by heartbeat and breathing. Therefore, tissue pulsation should be dampened as much as possible, for example, by covering or embedding the exposed organ with agar. Tight control of the anesthesia or artificial respiration can help to alleviate pulsation. For time-lapse imaging of cell structures, we recommend acquiring small subvolumes (image stacks of 10–20 focal planes) in anticipation of lateral or focal drift, which can then be corrected offline using correlation methods. Image acquisition can also be synchronized to the heartbeat by triggering individual frames using a simultaneously recorded electrocardiogram, to ensure that all images have the same phase relationship to the heart beat [34]. For long-term imaging over days to months, animals are multiply reanesthetized and image stacks of the same subvolume are acquired. In the brain, these experiments are performed either through the thinned skull [35–38] or through a
chronically implanted glass window [39–41] (Figure 14.3a). In cancer research, a dorsal skin-fold chamber with a glass window is used for imaging implanted tumors [42]. To repeatedly find the same structure (for example, a cell, a blood vessel, an amyloid plaque, or even a synapse), stable anatomical landmarks, such as the surface blood vessel pattern, can be used.

Functional imaging from neuronal dendrites in the intact neocortex has, so far, still required the introduction of synthetic calcium indicators via electrodes [24–26, 43–45], which has the advantage of providing additional in vivo intracellular electrophysiological data. The combination of two-photon optophysics with electrophysiology [46] will be crucial for studying synaptic integration and single-cell computation in living animals. Great hope still rests on the in vivo application of genetically encoded functional indicators [47–51].

However, for classical in vivo neurological observations with windows opened in the mouse brain, the presence of the cranium greatly increases the scattering and absorption of light during propagation. Figure 14.3a demonstrates the application of imaging on a thinner skull and the replacement of the skull with a thin slide by a surgical approach. However, we are currently unable to be clear whether this surgical procedure has an impact on neural activity in the brain of mice – it is common sense to infer that opening a window on the brain and thinning or even removing the skull would necessarily have a dramatic impact on the actions of the organism, making it an important obstacle to the application of two-photon excitation microscopy to in vivo imaging observations. This obstacle is also driving the development of tissue optical clearing techniques, making skull transparency an important and much needed developing direction.

Tissue optical clearing with TPEM
As mentioned earlier, conventional two-photon excitation imaging is affected by the scattering of light in the sample, even though the use of long wavelengths (second or even third harmonics) can enhance the penetration of the excitation light, resulting in a higher light intensity and less scattering of the excitation light irradiated on the sample, yet it remains powerless when faced with, for example, a centimeter-scale sample of a mouse brain. Sequential two-photon excitation (STP) in combination with extremely high-precision mechanical slices allows high-resolution imaging of intact mouse brains with submicron accuracy, but it poses the problem of long imaging times.

So the idea of combining tissue optical clearing techniques with the already well-established two-photon excitation imaging came naturally. As described in the previous sections, existing tissue optical removal techniques have made it possible to transparent the intact mouse brain, nervous system, and even the whole mouse, at which point, in combination with two-photon excitation imaging, the imaging depth of the sample will be greatly increased, limited only by the working distance of the excitation and detection mirrors, and in combination with the electromechanical displacement stage, stitching imaging in a single plane will be possible, and in the horizontal plane will no longer be limited by the sample size.

Taking the 2019 paper Reconstruction of 1,000 Projection Neurons Reveals New Cell Types and Organization of Long-Range Connectivity in the Mouse Brain by Janelia Farm’s Jayaram Chandrashekar research group published in Cell as an example [52], the researchers wanted to achieve an ideal submicron level resolution while observing information on the distribution of locations such as different brain regions through which long-distance projection neurons pass throughout the brain, which challenged both the resolution and sample size of the imaging. Using a well-established high-resolution two-photon excitation imaging system combined with the CUBIC tissue optical clearing method, the researchers achieved a 3D resolution of $0.3 \times 0.3 \times 1 \mu m^3$ to acquire a complete three-dimensional data of the whole mouse brain, and based on this data completed the localization and tracking of the specific labeled nerves. Figure 14.4 shows a schematic of the imaging with the results.

In addition to tissue optical clearing techniques combined with STP, researchers are now exploring ways to make the skull transparent. This is because, for in vivo imaging, both the chemical immersion and rinsing required by current tissue optical scavenging techniques result in the inactivation of the sample, resulting in the death of the organism itself, which in turn defeats the purpose and significance of in vivo observations. Therefore, it is a compromise solution for less impactful cranial hyaline. At the same time, more advanced tissue optical clearing technologies that are completely harmless to the organism itself are also being explored and researched in full swing. It is believed that in the near future, with the flourishing development of tissue optical clearing technology, two-photon excitation microscopy in vivo imaging and its combination will no longer be just a dream, and many life phenomena and problems in the organism, such as the propagation and regulation of calcium signals, will see greater breakthroughs.

Tissue optical clearing with LSFM
In the previous subsection, this book focused on the principles of traditional two-photon excitation microscopy methods and the problems faced by two-photon excitation microscopy methods when faced with the problem of light scattering and absorption in biological sample tissues. It concludes with a presentation on how tissue optical scavenging techniques can help solve problems and greatly enhance the scale of 3D imaging, opening a new door for more and broader research into biomedical problems.

This section will detail an emerging three-dimensional microscopic imaging method developed in recent years – light-sheet fluorescence microscopy. Similar to the structure of the previous section, the principle of the light-sheet fluorescence microscopy method is first described. Unlike the two-photon excitation microscopy method, due to the principle limitation of the light-sheet fluorescence microscopy method itself, it is only able to detect the signal at a depth of about $100 \mu m$ without the tissue optical clearing technique. Only when the two are combined can the advantages of light-sheet fluorescence microscopy imaging be fully reflected. This section will
present several of the more cutting-edge light-sheet imaging methods available today, demonstrating the different scales of research that have been conducted when they are combined with tissue optical clearing techniques, fully demonstrating the convenience that these two techniques provide to life science research today.

Fluorescence microscopy in concert with genetically encoded reporters is a powerful tool for biological imaging over space and time. Classical approaches have taken us so far and continue to be useful, but the pursuit of new biological insights often requires higher spatiotemporal resolution in ever-larger, intact samples and, crucially, with a gentle touch, such that biological processes continue unhindered. LSFM is making strides in each of these areas and is so named to reflect the mode of illumination: a sheet of light illuminates planes in the sample sequentially to deliver volumetric imaging. LSFM was developed as a response to inadequate four-dimensional (4D; x, y, z, and t) microscopic imaging strategies in developmental and cell biology, which overexpose the sample and poorly temporally resolve its processes. It is LSFM’s fundamental combination of optical sectioning and parallelization (Figure 14.5) that allows long-term biological studies with minimal phototoxicity and rapid acquisition.

FIGURE 14.4 Imaging pipeline. (a) Animals were injected in targeted brain areas with a combination of a low-titer AAV Syn-iCre and a high-titer AAV CAG-Flex-eGFP/tdTomato. (b) Two-photon microscope with an integrated vibratome. Inset: sequential imaging of partially overlapping image stacks. (c) Image stacks overlapped in x, y, and z. (d) Rendered brain volume after stitching. (e) Horizontal maximum intensity projection through a 1300 × 2000 × 3600 mm³ volume of the motor cortex containing labeled somata and neurites. Horizontal dashed lines mark physical tissue sections; vertical dashed line represents stack boundaries. Dashed box is region shown in (f). (f) Example of boundary region between two adjacent image stacks before (left) and after stitching (right). Dashed line indicates overlap region. (g) Residual stitching error in the lateral and axial directions.
Combination of tissue optical clearing

Principle of conventional LSFM

The starting point for the design of light-sheet systems should be a specific biological question or application. Indeed, LSFM is particularly suited for construction around the sample, because the decoupled illumination and detection paths of LSFM provide endless scope for customization and because the microscope may be arbitrarily arranged in space. Although commercial systems perform a crucial role in multiuser environments, the most exacting applications require custom solutions. The power of the custom approach becomes particularly apparent when the application pushes the limits of the technology, for example for high-speed in toto imaging of neural activity [53], cardiac dynamics [54, 55], gastrulation in whole embryos [56, 57], and physiologically representative subcellular imaging [58]. Likewise, where concessions must be made to sample-mounting protocols to allow normal development of physically sensitive embryos [59–62], geometric flexibility is crucial, whereas for behavioral studies the ability to spatially [63] or spectrally [64] avoid visually evoked responses may prove invaluable.

To understand how LSFM elegantly sidesteps many of the issues that plague conventional microscopies, consider the question: how does microscopy allow us to visualize biological tissues in 4D (x, y, z, and t)? A cursory appreciation of optics is sufficient to understand that out-of-focus objects appear blurred. This is why epifluorescence microscopy, which captures volumes as 2D projections, can only achieve high-contrast imaging in thin samples. The acquisition of images without somehow being able to discriminate based on depth reduces biological systems, which are three-dimensional without exception, to a planar representation. Just as tissue can be mechanically sectioned, sectioning can be achieved noninvasively by optical sectioning, which point-scanning confocal and multiphoton microscopies achieve by the removal of the out-of-focus signal and by confining excitation to the focal volume, respectively. However, in each case, the serial nature of the acquisition process limits the speed with which volumetric data can be collected. Also, crucially, regions that do not directly contribute to the useful signal are exposed repeatedly, which leads to photodamage.

The manner in which LSFM overcomes these limitations is remarkably simple. Taking a wide-field microscope as its basis, the sample is illuminated from the side with a sheet of light, ensuring that signal arises only from in-focus regions (Figure 14.5), thereby reducing the total exposure. A camera collects the resulting fluorescence signal, sequentially imaging the volume as 2D optical sections, thus parallelizing the imaging process within each plane. As such, the dwell time at each point is orders of magnitude higher than that in the point-scanned case, which allows for commensurate reductions in peak light intensity. Because the peak intensity and total power delivered will each have a bearing on photodamage rates, the combination of intrinsic sectioning (entire illuminated volume contributes to useable signal) and parallelization (plane-wise acquisition, millisecond exposure times) allows for gentle and rapid imaging. It is this combination of speed, 3D resolving power, and low phototoxicity that makes LSFM such an attractive imaging tool to confront a range of biological questions.

A historical perspective is useful in understanding some of the most fundamental choices that need to be made in building or choosing a light-sheet microscope. The first, at least in a form that would be recognizable to a modern user, was developed a little over a decade ago and called selective-plane-illumination microscopy (SPIM). SPIM (Figure 14.6) illuminates the sample with a static 2D light sheet focused by a cylindrical lens, and its use demonstrated for the first time that long-term fluorescence imaging of entire developing embryos could be achieved without unduly impairing their health. As a testament to the strength of SPIM, this fully parallelized scheme (simultaneous whole-plane illumination and detection) has yet to be improved upon for its use in long-term developmental imaging. However, a drawback of illuminating an entire plane at once from the side is the presence of striped artifacts in the...
resulting data, which arise from the refraction, scattering, and absorption of coherent light within tissue. In relatively transparent samples (such as zebrafish embryos), the effects are minimal, but in optically dense samples (such as fruit flies), they may be more severe. A later variant, multidirectional SPIM (mSPIM) (Figure 14.6) resonantly pivots the light sheet about its focus, illuminating more uniformly and thus reducing the stripe artifacts [66].

Digitally scanned light-sheet microscopy (DSLM) (Figure 14.6) provides the counter to the full parallelization of SPIM, sweeping out a virtual light sheet by scanning a Gaussian beam through the sample [67]. Because only part of the plane is illuminated at a given time point (i.e., the pixel dwell time decreases), the peak laser power delivered to the sample must increase proportionally to maintain the SNR, thus increasing the chances of fluorophore saturation and rates of photodamage. In optically dense samples, however, DSLM is superior in reducing striping, and the increase in intensity may be manageable even if not desirable (Figure 14.6b). All light-sheet microscopes are ultimately based on either the SPIM or DSLM architecture, and the choice of whether to scan or not can be crucial in balancing photodamage, imaging speed, and quality.

The lateral and axial resolution in light-sheet microscopy is determined slightly differently than that of conventional techniques. The product of the illumination and detection PSFs determines the axial resolution. Thinner, high-NA light sheets, therefore, provide superior axial resolution; however, diffraction dictates that a tightly focused (high-NA) Gaussian light sheet diverges rapidly away from the focus (Figure 14.7). Generally, the region over which the light sheet spreads by no more than \( \sqrt{2} \) times the thickness at waist is taken to demarcate the area that is useful for imaging. For a focused Gaussian beam, the resulting light sheet length, \( z_{ls} \), and the thickness, \( \omega_h \), are given as:

\[
z_{ls} = \frac{2\lambda}{\pi NA^2} \tag{14.3}
\]

\[
\omega_h = \frac{2\lambda}{\pi NA} \tag{14.4}
\]

The NA dependence demonstrates that there are diminishing returns on decreasing the light-sheet thickness in terms of the achievable FOV, and so typically some trade-off between usable FOV and axial resolving ability has to be made. Early implementations of LSFM focused on whole-embryo imaging for good reason: isotropic, subcellular resolution requires an ultrathin (high-NA) light sheet, which severely limits FOV.

The theoretically achievable lateral resolution is simply that of a wide-field microscope, governed by the wavelength and NA of the objective lens used for detection. For low magnification n–high NA detection lenses, undersampling is frequently employed to sacrifice resolution for field of view (FOV), and sCMOS (scientific complementary metal-oxide-semiconductor) cameras are generally favored since they deliver ~4-16 \( \times \) larger FOVs (by area) than typical EMCCD (electron-multiplying charge couple device) cameras for equivalent spatial sampling. For cases in which excellent light sensitivity is required, EMCCDs may offer a superior solution, notably for superresolution and multiphoton implementations. Moreover, under light-starved conditions, a high-detection NA is favorable, as the collection efficiency scales with NA [2]. However, another consequence of high NA is that the DOF of the objective lens will be small, and so only a thin slice of the sample remains in focus. Usually the light sheet has to be thicker to cover the FOV, which compromises the sectioning ability and lowers the contrast.

### Three challenges and corresponding methods for LSFM

The first challenge for achieving high-resolution imaging in LSFM is largely a geometric issue. High numerical aperture (NA) detection optics are favorable for light-collection
Combination of tissue optical clearing

- Efficiency and lateral resolution, whereas high-NA illumination produces thinner light sheets, yielding superior axial resolution and sectioning ability. Unfortunately, concurrent high NA in both pathways is sterically constrained as high-NA lenses are, by necessity, bulky. Although the size of the objective lens, which dictates the achievable NA, requires little consideration in an “epi” configuration, trying to position high-NA (>0.9) water-immersion lenses such that their orthogonally oriented foci overlap is a fruitless task. Regardless, for many applications, the light-sheet NA (which governs the light-sheet length and thickness) may be much lower, for example, 0.06 > NA > 0.02 is typical to cover a field of view (FOV) of 50–500 µm (λ0 = 488 nm). This permits the use of ultralong working-distance, low-NA lenses, substantially relaxing the constraints on the choice of objective lens for detection. A summary of objective lenses that are typically used for illumination and detection in LSFM is given as a guide to what can be orthogonally coaligned. It is worth noting that some particularly advanced systems have used custom-designed objectives [53, 68]; however, the cost and complexity may be prohibitive for the majority of microscopists.

- In principle, LSFM provides an ideal platform for far-field superresolution imaging. In a live-cell context, in particular, the LSFM approach is beneficial relative to near-field techniques (for example, total internal reflection fluorescence microscopy), which image molecules located within one wavelength of the coverslip. Localization-based techniques exploit the photophysics of molecular probes and allow spatial resolution of tens of nanometers. However, in thick, living samples, the indiscriminate nature of the illumination and the numerous exposures required to stochastically construct the image lead to photodamage and out-of-focus signal. However, all the aforementioned have a precondition: the tissue optical clearing. Without the clearing techniques, because LSFM uses normal lasers, the scattering and attenuation caused by biological samples prevent the propagation of excitation light. By confining the illumination to a thin plane, both can be ameliorated, but it still penetrates for a depth of only about 100 µm. At the same time, the emission fluorescence is also unable to spread to the superficial layer and be collected by detection objective lens.

- In spite of the mutual exclusivity between high detection and illumination NA, a number of localization-based superresolution light-sheet fluorescence microscopes have been reported. The individual molecule localization selective-plane illumination microscope (IML-SPIM) provides a prime example with a high NAill (1.1) limiting NAdet (0.3) [69]. Although axial localization is achieved through depth-dependent astigmatism [70], the relatively thick light sheet compromises sectioning. Naturally, as one ventures to smaller feature sizes or exceptionally low light levels, the required increase in detection NA eventually becomes a bottleneck, and alternative geometries have to be sought.

- To overcome this limitation, Gebhardt et al. used a pair of vertically opposed objective lenses with a 45° mirrored cantilever to redirect a thin light sheet (full-width at half maximum [FWHM] thickness of 1 µm) horizontally into the detection plane (Figure 14.8a), permitting the use of an ultrahigh-NA detection objective lens (NAdet,max of 1.4 in oil) [71]. This reflected light-sheet microscopy (RLSM) provides an additional benefit, as the inverted imaging geometry facilitates construction around conventional microscope bodies. Single-objective SPIM (soSPIM) launches the light sheet from the detection objective lens (NAdet of 1.4 in oil) (Figure 14.8b). In this case, a microcavity mirror coupled to the sample support is used to horizontally reflect the light sheet and deliver similar sectioning to that by RLSM [72]. Because the illumination and detection planes are coupled in soSPIM, the production of volumetric data is more complex and requires a combination of scanning and refocusing. To change the axial position of the light sheet, the beam is swept laterally across the mirror while the detection plane remains coaligned by translating the objective lens. Naturally, this is accompanied by a shift in the light-sheet waist across the FOV. To compensate, an electrically
An elegant solution that avoids a reflective element is the so-called π-SPIM (Figure 14.9), which uses a pair of nonorthogonal objectives (90 < θ < 180) and oblique illumination to relax mechanical constraints somewhat [74], in essence forming a two-objective variant of a highly inclined and laminated optical sheet (HILO) microscope [75]. Although the obliquity sacrifices some of the illumination objective NA, the geometry allows a combined illumination and detection NA near the theoretical maximum, using only off-the-shelf components (NA_{ill} = 0.71; NA_{det} = 1.1 (water); fill factor = 0.986).

The second challenge in achieving high-resolution imaging with LSFM is to maintain high axial resolution over a large FOV. Because the overall or system point-spread function (PSF) arises from the overlap of illumination and detection PSFs, isotropic resolution is achievable, in principle, by sufficient axial confinement of the light sheet [76]. Maintaining a thin light sheet across a FOV > 10 μm is problematic, however, as a high-NA Gaussian light sheet spreads rapidly away from the focus. Consequently, the most common light-sheet variants fail to achieve a truly isotropic PSF, and axial resolution is typically no better than ~1 μm (or between two and three times worse than lateral resolution). Although this is perfectly adequate for cellular resolution, it may be limiting for cases in which subcellular resolution is required across a large FOV.

A number of solutions to this problem, using nondiffracting beam modes, have emerged, the most common being the Bessel beam, whose cross-section consists of a narrow central core surrounded by a series of rings of diminishing intensity (Figure 14.10). These beams are governed by diffraction like any other beam, but they maintain an invariant profile over many times the Rayleigh range of a Gaussian beam of equal NA. Planchnon et al. used a moderate NA (0.8) in both the illumination and detection pathways to deliver ~300 nm isotropic resolution over a FOV spanning 40 μm along the propagation axis of a scanned Bessel beam [77] (Figure 14.11). Bessel beams have been shown to offer measured improvements in a turbid medium, penetrating 1.55x further into human skin.
Combination of tissue optical clearing

Although impressive, the contrast-limiting effect of the concentric side lobes and associated additional photon load discussed earlier are similarly applicable. This may be ameliorated by sectioning the Bessel beam in a manner such that the ring structure is suppressed above and below the imaging plane while maintaining the self-reconstruction ability, although this broadens the resulting light sheet. However, the beam side lobes illuminate out-of-focus regions, which compromises sectioning ability, degrades contrast, and unnecessarily exposes the sample. By combining with optical-sectioning structured illumination (OS-SIM) or two-photon excitation [58, 80], this out-of-focus
contribution to the image can be removed or suppressed, respectively, with the caveat that the additional exposures or pulsed illumination increase rates of photobleaching [80]. Additionally, the OS-SIM algorithm produced reconstruction artifacts and discarded a large portion of the useful signal [77]. Fei et al. used the electronic rolling shutter of sCMOS and the confocal line detection effect helped to reject the effect of side lobes, which achieved subcellular resolution at 1-µm range FOV [81]. Gao et al. later adapted the technique to utilize super-resolution structured-illumination algorithms (SR-SIM) to provide an improvement in resolution of between 1.5-fold and 1.9-fold, an increased SNR, and a more judicious use of the photon budget [80]. By using multiple Bessel beams in parallel, a commensurate reduction in peak intensity was possible, further reducing photodamage.

Given the correct periodicity, a linear array of Bessel beams may interfere destructively, such that the rings are somewhat suppressed, producing an optical lattice. This realization led to the development of lattice light-sheet microscopy (LLSM), which is capable of delivering ultrathin (FWHM 1 µm) light sheets in a highly parallelized manner [68] (Figure 14.12). The optical efficiency afforded by this approach coupled with a NA_{det} of 1.1 allowed the electron-multiplying charge couple device (EMCCD) to be replaced with a faster scientific complementary metal-oxide semiconductor (sCMOS) camera. Along with low magnification (25x), the result is a larger FOV than previous Bessel beam implementations [77, 80] (~80 × 80 µm) and a plane-wise imaging rate as high as 200 or 1,000 fps [68] for multi- and single-color imaging, respectively. With the increased detection NA, custom illumination optics were required to maximize the available angular space, delivering a NA_{ill,max} of 0.65.

Other pseudo nondiffracting beams also exist. The Airy mode has been shown to yield thinner light sheets over larger FOVs than Gaussian or Bessel beams of comparable NA [83]. Unfortunately, to allow the beam side lobes to contribute positively to image formation, the data must be deconvolved, requiring that the Airy beam side lobes remain in focus. In turn this has limited the detection NA to 0.4, which is counter to the pursuit of high resolution. For now, the Airy beam remains largely a curiosity, and future studies in a more demanding biological context are welcomed.

Rather than use complicated beam shaping, high axial resolution and large FOVs can be achieved by sweeping a moderate-high NA Gaussian beam through the sample along the propagation axis. Effectively, this approach shares the focus temporally between different focal depths to produce a long and thin light sheet, while sacrificing some (1D) parallelization relative to the analogous SPIM- or DSLM-based approach. Dean, Fiolka, and Fei et al. have used ultrasonic lenses or voice coil motor to sweep a focused beam through the sample to achieve sheet thicknesses (FWHM) of 465 nm and 1.7 µm over FOVs of 50 µm and 3.3 mm, respectively [83–85]. Dean, Fiolka [84], and Fei [85] adopted confocal line detection (CLD), which effectively captures the 2D image line-wise to remove out-of-plane contributions from the beam tails (Figure 14.13a). Relative to DSLM, the 2D-scan or sweep process and associated decrease in dwell time required higher intensities still, which spurred the development of axially swept light-sheet microscopy (ASLM). In 2019, the new...
Combination of tissue optical clearing

ASLM represented by ctASLM [86] and mesoSPIM [88] further applied this technology to whole mouse brain imaging, bringing new opportunities for the imaging of large-scale biological samples.

In one sense, ASLM is akin to SPIM, producing a short depth-of-field (DOF) light sheet with cylindrical optics (Figure 14.13c). ASLM sweeps the short light sheet through the sample using a remotely situated objective lens and swept piezo mirror for aberration-free refocusing [88]. Like DSLM, this approach also produces a virtual light sheet in one dimension. ASLM produces ultrathin light sheets over large FOVs by using CLD to remove fluorescence from the beam tails, delivering comparable resolution to LLSM without the need for complicated processing or reconstruction. Illuminating out-of-focus regions is, however, more wasteful with the photon budget.

The stability and structure of the Bessel beam makes it an ideal candidate for confocal treatment, as the beam penetrates further into tissue without being unduly perturbed. Fahrbach et al. explored the depth-dependent attenuation of signal for Gaussian and Bessel beams, demonstrating that the decay in signal with depth is more severe for the former [89]. It is also worth bearing in mind that the spatial filter is only 1D and so will not remove light that is scattered along the slit axis. Silvestri et al. noted that this causes an increase in background signal at depth while the useful signal decreases [90]. CLD requires no additional exposures or postprocessing steps and has become a widespread and powerful tool for scanned LSFM systems. Recent developments include beam multiplexing to utilize the twinned rolling shutters of the current generation of sCMOS cameras, which delivers higher imaging speed [91] as well as more complex multiview implementations [53, 92].

Relative to conventional microscopies, LSFM performs remarkably well at low-to-moderate NA (owing to both general efficiency and axial resolving power derived from overlap of orthogonal illumination and detection PSFs). Correspondingly, samples can be positioned far from mechanically small, long-working-distance objective lenses to allow easy manipulation of, and unparalleled optical access to, the sample. Even so, the speed of LSFM is crucial in allowing multiview imaging; conventional methods are simply too slow to take advantage of the paradigm, even when geometry allows it.

The first aspect of multiview imaging concerns improved axial resolution. Because axial resolution is typically lower than lateral, imaging from two directions (separated by 90°) will produce two data sets, which, taken together, sample all axes with the best possible resolution. The different viewing angles can subsequently be combined to produce a single data set with improved axial resolution [93]. A number of multiview registration [94] and deconvolution algorithms exist, with the most powerful ones capable of performing real-time processing [95] and requiring fewer views [96]. Two views are insufficient to provide truly isotropic resolution; however, additional views reduce the temporal resolution, unnecessarily expose the sample, and encode increasing amounts of redundant information [97].

Large and opaque samples may additionally benefit from the superior sample coverage offered by multiview imaging. Full optical coverage can be achieved, even on distal sides of the embryo, by sequentially recording image stacks from different viewing angles and computationally fusing them to produce a single high-resolution data set. For example, consider an embryo with a degree of rotational symmetry, such as the ellipsoidal Drosophila embryo or the spherical zebrafish embryo. In addition to the small-scale imaging of embryos, multiview imaging is more widely used in large-scale imaging. Due to the weak penetration of the Gaussian light sheet we mentioned, with multiview imaging, it can completely penetrate the sample deep layer, so only needs to penetrate half of the depth to complete the imaging. With the deconvolution algorithm [98], the original details of the sample can be completely restored. As shown in Figure 14.14, the light sheet and imaging optics overlap to provide good optical coverage of a quarter of the embryo; therefore, by taking four views that are spaced by 90° each, the entire sample can be covered. Acquisitions from a few closely spaced angles can also help when the sample exhibits a complex geometry that may be changing during a time-lapse experiment, as these views increase the chance to capture a critical event from the best possible angle.

Multidirectional SPIM (mSPIM) was the first technique to add a second illumination lens, which effectively provides two views of the sample as it is sequentially illuminated from either side [66]. Although synchronous double-sided illumination is possible, this results in a loss of contrast as the light sheet spreads toward the opposing side of the embryo. Because a rudimentary fusion of the two views can be achieved by stitching together the good half of each image, only half of the FOV needs to be covered by each light sheet. As such, each light sheet may be made thinner by a factor of \( \sqrt{2} \) without compromising the FOV. Nevertheless, full optical coverage still requires at least one rotation, as half of the embryo remains inaccessible to the single detection path.

The latest IsoView allows simultaneous illumination and detection in all four paths, and eliminates crosstalk either spatially, by using phase-shifted confocal line detection; or spectrally, by switching between colors in the orthogonal pathways [53]. Both modes require scanning all four objectives...
Multiview imaging. (a) Improved axial resolution can be achieved by reconstructing images taken from different angles, either achieved by sample rotation (two, three, or four lenses) or by using all of the lenses for illumination and detection (4x). (b) Optical coverage arises from the overlap of efficiently illuminated and detected quadrants. The (minimum) number of imaging angles to provide full optical coverage and improved axial resolution is given for each case.

To refocus the corresponding detection plane, with the result being that the beam waist of each light sheet is translated an equal distance. Correspondingly, the light sheet is more weakly focused to span the additional distance, compromising sectioning somewhat. Nevertheless, multiview deconvolution produced data sets with a spatial resolution of 2.5 µm or better, even in the center of the Drosophila embryo.

Though not specific to LSFM, refractive-index matching by chemical clearing of tissue finds a natural home in this context, allowing for exceptionally large, fixed samples to be imaged with microscopic resolution and in a reasonable period of time. Nevertheless, the transition to larger sample sizes does provide some unique optical challenges, and although clearing makes even deep tissues accessible, without corresponding changes to the optical components, they remain tantalizingly out of reach. Dodt et al. reported an ultramicroscope that uses low magnification and NA optics to image cleared mouse brains over centimeter-sized FOVs [99] naturally, with some sacrifice to spatial resolution. The generation of thinner, less-divergent light sheets benefits subcellular and macroscopic LSFM imaging alike. Saghafi et al. were able to shape the illuminating light sheet using several aspheric and cylindrical lenses in series to produce light sheets with a 4-µm thickness at the beam waist and with little divergence over several millimeters [100]. Others have used binary-pupil phase masks to achieve similar results [101, 102]. Tomer et al. adopted a different approach to imaging optically cleared tissues in the CLARITY optimized light-sheet microscope (COLM) [103], which tiles the acquisition process to cover large FOVs. The superior collection efficiency afforded by high-NA optics compensates somewhat for the additional exposures by making better use of the available light, whereas the relatively high magnification and NA affords submicron resolution. To compensate for misalignment caused by residual refractive index inhomogeneities deep inside tissue, an autocalibration routine adjusts the light-sheet position such that the two planes maintain coalignment throughout the volume.

The third challenge is to achieve high imaging throughput while maintaining high 3D resolution. The aforementioned methods such as ASLM and multiview light sheet imaging all need to improve the conventional light sheet imaging system, such as scanning with a galvanometer, using the CLD effect to reject the side lobes of the Bessel beam or reject the part out of Gaussian light sheet’s Rayleigh range. As for multiview imaging, a rotating motor can be used to rotate the sample and acquire information from multiple angles, and then an algorithm can be used to fuse it to a whole 3D volume.

The main problem of these methods is to reduce the imaging frame rate, which in turn reduces the imaging throughput. ASLM is affected by the response and movement time of the galvanometer or ETL, and due to the use of sCMOS electronic rolling shutter, the maximum frame rate of the camera will be reduced by half, which greatly limits the total optical throughput of the imaging system. Similarly, multiview imaging cannot complete the three-dimensional scanning of the sample at a fixed angle of view. The more precise the angle divided during the acquisition process, the higher the resolution and the longer the acquisition time. The time required for four angles is four times that of single-sided illumination, while the time required for eight angles will be further doubled.

The key to the improvement is to combine light sheet fluorescence microscopy with the resolution enhancement algorithm, achieving that under the combination of a thicker light sheet excitation and a lower magnification detection objective, and acquiring the raw data with low three-dimensional resolution with extremely high throughput. After acquisition, the three-dimensional resolution enhancement algorithm can be used to enhance 3D resolution of raw data to obtain high-resolution
Combination of tissue optical clearing

data comparable to data acquired by the combination of a thinner light sheet excitation and a higher magnification detection objective. The most commonly used algorithm is deconvolution. If the point spread function (PSF) of the optical imaging system is known, the PSF can be used to complete the deconvolution operation. The commonly used algorithms are the Lucy-Richardson algorithm and Wiener filtering. Deconvolution is simple and convenient, but the disadvantage is that the effect is limited. For images that are too blurry, it is difficult to achieve the desired resolution enhancement effect. In the research work of Fei et al. [98], the deconvolution algorithm and the subvoxel-resolving (SVR) algorithm are combined to achieve better enhancement effects. With the multiview imaging at four times, a complete mouse brain was observed within half an hour, and achieved a three-dimensional resolution of approximately 1.5 μm (Figure 14.15), overcoming the contradiction between high throughput and high resolution.

Several other commonly used algorithms in superresolution research, such as SIM, require the synthesis of multiple raw images and therefore cannot meet the requirement of increasing the acquisition throughput. Compression sensing, which is also based on a single raw image, is also favored by researchers. Compressed sensing is based on sparse raw signals, allowing image acquisition at sampling rates lower than the Nyquist sampling frequency, while for dense signals, images can be converted to sparse domain by Fourier transform, etc. to recover, with equally impressive effects. Therefore, researchers reduced the imaging time in two ways: the first is by directly reducing the exposure time during the acquisition of single-frame images to obtain the original image with low signal-to-noise ratio and then using the compressed sensing to recover the results of high SNR [104, 105]; the second is to use the same thicker light sheet and low-magnification detection objective lens, and after obtaining the blurred image, use the compressed sensing to enhance the 3D resolution [81]. Both methods can increase the imaging throughput, and due to the more pronounced effect of 3D resolution, the second method can increase the throughput up to 64 times, achieving whole mouse brain data acquisition within minutes, which can be recovered to ~1 μm isotropic resolution (Figure 14.16).
Conclusions and outlook

This chapter respectively introduces the principles of traditional TPEM and LSFM and the problems of light scattering and absorption when imaging biological tissues. The main strength of TPEM and Bessel light sheet fluorescence microscopy techniques is the ability to maintain resolution and contrast within scattering tissue. The long wavelength and concentric circle structure formed by interference ensure that when they penetrate thick biological tissues, they can better avoid the influence of absorption and keep their shape unchanged. Even so, the penetration depth is still unsatisfactory when facing opaque biological samples.

The emergence and development of tissue optical clearing technology successfully extended the imaging size to several millimeters, even centimeters. For the study of in vitro imaging, this combination provides great convenience for the whole brain nerve observation and other fields. At the same time, LSFM can also use the two-photon long wavelength as an illumination laser source, which can further enhance the penetration depth. With the current variety of new modified light sheets, such as multiview imaging and ASLM, it can achieve high-throughput, high-resolution imaging, which can image a whole mouse brain in several hours with cellular 3D resolution. At the same time, for small samples at the single cell level, submicron three-dimensional resolution can also be observed.

Even though there is currently a shortage of applications for in vivo imaging, we still believe that as tissue optical clearing technology continues to evolve, new harmless and rapid clearing techniques will inevitably emerge in the future that can make tissues transparent without harming the activity of the organisms themselves. A new revolution will also take place in the field of live imaging at that time.

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