Topic Introduction

Temporal Focusing Microscopy

Dan Oron and Yaron Silberberg

Axial localization of multiphoton excitation to a single plane is achieved by temporal focusing of an ultrafast pulsed excitation. We take advantage of geometrical dispersion in an extremely simple experimental setup, where an ultrashort pulse is temporally stretched and hence its peak intensity is lowered outside the focal plane of the microscope. Using this strategy, out-of-focus multiphoton excitation is dramatically reduced, and the achieved axial resolution is comparable to line-scanning multiphoton microscopy for wide-field excitation and to point-scanning multiphoton microscopy for line excitation. In this introduction, we provide a detailed description of the considerations in choosing the experimental parameters, as well as the alignment of a temporal focusing add-on to a multiphoton microscope. We also review current advances and applications for this technique.

INTRODUCTION

The ability to perform optical sectioning is one of the great advantages of laser-scanning multiphoton microscopy and photoactivation systems. This introduces, however, a number of difficulties caused by the scanning process, such as lower frame rates caused by the serial acquisition process. Whenever these problems need to be circumvented, temporal focusing multiphoton microscopy makes it possible to confine multiphoton excitation to a single plane completely without scanning. The method relies on utilization of geometrical dispersion to compress the ultrashort excitation pulse as it propagates through the sample, reaching its shortest duration at the focal plane, before stretching again beyond it. This introduction outlines the requirements and the limitations of this technique and presents a brief summary of recent applications, including scanningless depth-resolved microscopy, improved depth resolution in line-scanning multiphoton microscopy, and confined photoactivation within a single two-dimensional plane.

AXIALLY RESOLVED MICROSCOPY

The confocal microscope, invented by Minsky more than 40 years ago (Minsky 1961), marked the dawn of an era of significant departure from the traditional principles of optical microscopy. In this new generation of microscopes, unlike their more traditional counterparts, an optical image of the sample is formed by scanning the sample point by point with a tightly focused illumination beam. The scattered laser light, or, more commonly, fluorescence induced by it, is detected through a confocal pinhole and collected to form an image. The main advantage of confocal microscopes is their optical sectioning capability. The introduction of multiphoton optical processes as imaging modalities in laser-scanning microscopy offers another mechanism for obtaining optical sections (Sheetz and Squier 2009, and references therein). Because of the nonlinear dependence of the signal on the illuminating

Adapted from *Imaging: A Laboratory Manual* (ed. Yuste). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

Cite this introduction as *Cold Spring Harb Protoc*; doi:10.1101/pdb.top085928

electric field, multiphoton scattering is observed only from regions in which the excitation field peaks (i.e., from the focal volume). This inherent mechanism for rejection of out-of-focus scattering eliminates the need for a confocal pinhole in the detection path. To date, a variety of multiphoton processes has been used for imaging, including both incoherent processes such as two-photon and three-photon fluorescence and coherent processes such as second-harmonic and third-harmonic generation and coherent Raman processes (see **Principles of Multiphoton-Excitation Fluorescence Microscopy** [Denk 2007]; **Monitoring Membrane Potential with Second-Harmonic Generation** [Wilson et al. 2014]; **Coherent Raman Tissue Imaging in the Brain** [Saar et al. 2014]). More recently, multiphoton photoactivation has become an important tool in biosciences (Nikolenko et al. 2007), enabling either controlled uncaging of a biologically active molecule in a particular spot within the specimen or as a tool for switching fluorescent molecules between an emissive and a nonemissive state. In the proper configuration, the latter method can enable subdiffraction-limited localization of the fluorophores.

In both confocal and multiphoton microscopes, however, optical sectioning capability comes at a cost—not all points in an image are simultaneously illuminated. For imaging purposes, this results in longer image acquisition times: in the range of a few tens of milliseconds per section with current commercial systems. When photoactivating caged molecules, serial excitation limits the number of points at which uncaging can occur within a given time window. This is an inherent limitation of laser-scanning microscopy that significantly limits its utility for imaging and control of fast dynamics.

Most methods developed to increase the image acquisition rate in multiphoton microscopes rely on illumination of more than one point at a time or "space multiplexing." As long as the excited points are sufficiently far from each other, depth resolution will not be significantly affected. The simplest example is the use of single-axis scanning combined with line illumination (Brakenhoff et al. 1995). The primary disadvantage of such a simple implementation is degradation in depth resolution. Rotation of a lenslet array is a more complex implementation of single-axis scanning that, when using a properly designed optical element, does not compromise depth resolution (Bewersdorf et al. 1998). Multiple-point illumination can also be accomplished using beam-splitter arrays (Fittinghoff et al. 2000; Nielsen et al. 2001) and digital holography (Sacconi et al. 2003).

TEMPORAL FOCUSING: THE WORKING PRINCIPLE

The use of a pulsed excitation field as in multiphoton microscopy offers yet another degree of freedom for improving the depth resolution in space-multiplexed microscopy—the temporal degree of freedom.

Taking into account the temporal shape (more specifically, the duration) of the excitation pulse, it is, in principle, possible to imagine a multiphoton microscopy scheme in which time and space exchange roles: The sample is illuminated by a pulsed plane wave whose duration becomes shorter as it propagates, reaches a temporal focus at the focal plane of the temporal lens, and then increases by further propagation. This scheme is shown in Figure 1A along with the conventional spatial focusing technique. Such a scheme can be used to achieve axially resolved excitation even for simultaneous illumination of the entire plane (Oron et al. 2005).

Optical Setup for Temporal Focusing

The experimental apparatus functions as a lens in which time and space exchange roles. A perfect lens focuses light along the directions perpendicular to the propagation direction without affecting its temporal profile (i.e., the duration of a light pulse is maintained along the propagation). By analogy, a pulsed optical field passing through the time lens is temporally compressed as it propagates, reaching its shortest duration (and highest peak intensity) at the temporal focal plane, before stretching again beyond it. This should be performed while in effect maintaining the spatial profile of the pulse. For

 Cite this introduction as *Cold Spring Harb Protoc;* doi:10.1101/pdb.top085928

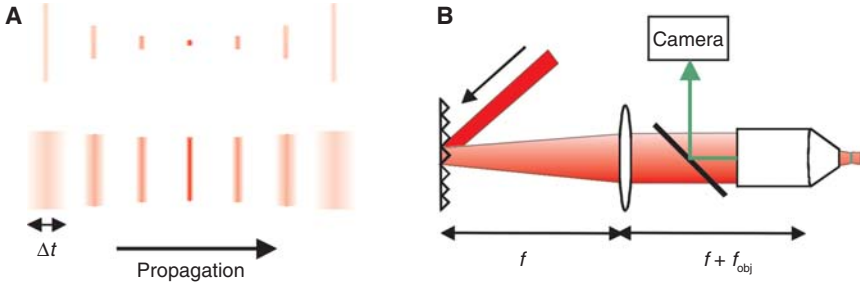

FIGURE 1. Temporal focusing microscopy. (*A*) The concept of temporal focusing. In standard point-scanning multiphoton microscopy, the beam is focused to a point (*top*), where the intensity is high enough to generate nonlinear scattering. In a temporally focused beam (*bottom*), the pulse reaches its shortest duration at the focal plane, so that the multiphoton signal is generated preferentially at the entire focal plane. (*B*) The optical setup for temporal focusing microscopy. A short-pulse laser beam is scattered by a grating and is demagnified by a telescope into the specimen.

this to be practical, the depth of the temporal focus should be as short as several micrometers, implying that dispersion has to be of geometrical origin. The setup for this, shown in Figure 1B, is closely related to the 4f grating-based pulse compressor, a common device in ultrafast optics applications (Oron et al. 2005).

The setup comprises a grating imaged onto the sample by a high magnification telescope made with an achromatic lens and a microscope objective. The grating is illuminated by an ultrafast laser beam at an angle that enables the laser's central frequency to be scattered toward the optical axis of the telescope. At the back focal plane of the objective, the pulse is separated into its constituent frequency components (colors). After the objective lens, each frequency propagates at a different angle, which introduces a relative phase shift between them because of propagation. It is only at the focal plane of the objective that all frequency components are in phase, reconstructing the original short pulse. Where it is out of focus, the relative phase is quadratic in frequency, corresponding to group velocity dispersion (GVD). Hence, outside the Rayleigh range of the objective lens, the pulse is temporally stretched, leading to weaker multiphoton excitation. For microscopy applications, the two-photon fluorescence image can be epidetected and imaged onto a camera. An example of such a set of axially resolved images of a *Drosophila* egg chamber, obtained without any scanning, is given in Figure 2.

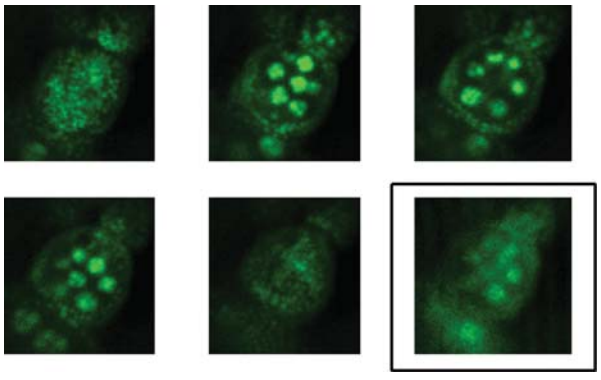

FIGURE 2. Scanningless depth-resolved images of a *Drosophila* egg chamber stained with DAPI (4′,6-diamino-2-phenylindole), a fluorescent DNA-binding probe. The images are optical sections of a *Drosophila* egg chamber containing 15 nurse cells and a single oocyte and wrapped by a layer of follicle cells. The images move spatially from the bottom of the egg chamber (*upper left* image) to its top (*lower middle* image). The area of each image is 140 × 140 µm. Images are separated by 7.5 µm. The *lower right* image shows a standard wide-field two-photon excited fluorescence image, to be compared with the temporally focused image directly above it, both focused at the same depth. Although some detail can be seen in the standard image, strong out-of-focus background dominates; a background that is practically eliminated in the temporally focused image.

Axial Resolution in Temporal Focusing

Temporal focusing has been shown to be equivalent, in terms of the axial response, to line focusing (i.e., spatial focusing along a single spatial dimension). For both coherent and incoherent multiphoton microscopies, this shows a full width at half-maximum (FWHM) axial resolution worse only by less than a factor of 2 but with a significantly increased signal at the tails. This applies, however, only to the case of a uniformly illuminated grating.

Improvement in the axial response can be achieved by combining temporal focusing with spatial focusing along one axis (Tal et al. 2005; Zhu et al. 2005). The end result, in this case, is an illuminated line at the image plane while the axial resolution of point-scanning microscopy is maintained. In practice, the simplest implementation is by illuminating the grating with a single line perpendicular to the grating grooves. Alternatively, the achromat of Figure 1B can be replaced with a cylindrical lens of a similar focal length.

In general, the axial response depends on the details of the illuminated pattern on the grating. For example, if the illumination pattern consists of several parallel lines, the axial resolution varies between that of point scanning and that of line scanning, depending on the distance between the lines. A full analysis of the effect of the excitation pattern on the axial resolution can be found in Papagiakoumou et al. (2009).

Choice of System Parameters for Temporal Focusing

To implement temporal focusing, care has to be taken to choose the right combination of grating, lens, and objective to fit the laser pulse parameters. Because, as in standard laser-scanning microscopy, the axial resolution stems from the spread of illumination angles at the focal plane, it is essential that the excitation pulse spectrum fill the back aperture of the objective, having a diameter d_{obj}. This dictates a relation between the pulse bandwidth $\Delta\lambda$, the groove density of the grating, k_g, and the focal length of the achromat, f:

$$\frac{f \cdot \Delta\lambda \cdot k_g}{d_{obj}} \approx 1.$$

For a typical 100-fsec Ti:sapphire oscillator, for which $\Delta\lambda \sim 10$ nm, and assuming $k_g = 1200$ lines/mm and $d_{obj} = 6$ mm, this corresponds to $f = 50$ cm. Because the pulse spectrum varies smoothly, the axial resolution only weakly depends on small deviations from the value of unity.

Once the focal length of the achromatic lens has been set, one needs to determine the required illuminated area on the grating. Because the system presented in Figure 1B is essentially a magnifying telescope, the illuminated area is simply smaller by a magnification ratio $M = f/f_{obj}$ than that on the grating. Again, for the above system parameters, with $f = 50$ cm, this ratio corresponds to about three times the objective magnification. The maximal illuminated area is determined by the total available laser power and the required excitation power per diffraction-limited spot. For typical values—1 mW average power per spot and 1 W of laser power—about 1000 points, equivalent to a 30×30 array, can be simultaneously illuminated without inducing a significant increase in image acquisition times. For larger illuminated areas, the required acquisition time will scale quadratically with the imaged area.

One convenient outcome for the temporal focusing setup is that dispersion precompensation of the excitation pulse can be performed using the same setup. Because the entire system is an asymmetric pulse compressor, a shift in the axial position of the grating introduces GVD, axially shifting the position of the temporal focus. For optimal imaging, the temporal focal plane has to coincide with the focal plane of the objective. To test this, a thin fluorescent sample should be axially scanned through the focal plane. When the grating is placed in the optimal position, the axial fluorescence response will be symmetric to small deviations from this position.

Line-Scanning Temporal Focusing

In line-scanning temporal focusing, the axial resolution is comparable with standard point-scanning multiphoton microscopes. This is an extremely useful and simple method for covering large areas. As

 Cite this introduction as *Cold Spring Harb Protoc;* doi:10.1101/pdb.top085928

discussed above, for typical laser parameters, the aspect ratio of the illuminated line can readily be as high as 1000, which, when combined with scanning along the orthogonal direction, covers a significant portion of the objective field of view. As discussed above, illumination of a single line can be achieved by replacing the achromat with a cylindrical lens (Zhu et al. 2005). For scanning, however, it is more beneficial to illuminate the grating with a line and to scan its position on the grating. This is readily achieved by a 2*f* combination of a galvanometric scanner and a cylindrical lens (Tal et al. 2005). The focal length of the cylindrical lens f_{cyl} is determined by the ratio of the laser beam diameter d_{laser} and the objective back aperture, such that

$$f_{cyl} = \frac{f \cdot d_{laser}}{d_{obj}},$$

ensuring that the objective back aperture is filled along both axes. In line-scanning temporal focusing, it is essential that the spatial focus and the temporal focus spatially overlap for optimal axial resolution. This may require more delicate alignment. In addition, achievement of optimal axial resolution may require external dispersion precompensation, unlike the case of plane illumination. Alternatively, the cylindrical lens should be slightly axially shifted to overlap the two foci. Using this setup along with a sufficiently strong fluorescent signal, video-rate axially resolved imaging can be readily achieved.

Generation of Arbitrary 2D Excitation Patterns

For a variety of applications, particularly photoactivation of caged compounds, it is useful to illuminate an arbitrarily shaped region in an axially resolved manner for the purpose of spatially confining multiphoton excitation to a single cell (Lutz et al. 2008). Because, in temporal focusing, the illumination pattern on the grating is practically imaged (and magnified) in an axially resolved manner onto the sample, the task is practically reduced to that of generating the corresponding magnified image on the grating. This requires conversion of the near-Gaussian spatial mode of the laser to an arbitrary shape (Papagiakoumou et al. 2008).

The problem of conversion from one intensity pattern to another is a classical one in Fourier optics (Goodman 2005). The best known solution is to place a 2D phase spatial light modulator (SLM) in the back focal plane and the grating in the front focal plane of a lens and to use an iterative algorithm (such as Gerchberg–Saxton) to generate the required pattern. Alternatively, a 2D amplitude SLM can be imaged onto the grating. The latter approach results in some speckle pattern in the image as well as slightly reduced axial resolution. The former approach, depending on the required shape, wastes more laser excitation power. If the presence of speckle interferes with the application, it can be smoothed by the introduction of a rotating diffuser placed in proximity to the grating.

Image Detection in Temporal Focusing Microscopy

Temporal focusing is an imaging modality that can be used in conjunction with any multiphoton microscopy technique, either coherent or incoherent. By far, the most common imaging modality is two-photon fluorescence. In this case, fluorescence from the excited plane is most easily collected by epidetection and a camera. The choice of camera should be determined by the nature of the measurement. Electron-multiplying charge-coupled devices (CCDs) are optimal for imaging fast dynamics at low light levels. For slower imaging or higher levels of signal, cooled CCDs are probably the best choice. Because the signal is detected by an imaging detector, it should be noted that, unlike with point-scanning multiphoton microscopy, the image quality deteriorates on scattering in the sample. Hence, temporal focusing is inappropriate for imaging deep into scattering tissue.

For coherent multiphoton imaging modalities, such as harmonic generation or coherent Raman scattering, the generated signal is mostly directed in the forward direction. Hence, the sample should be placed between two objective lenses. Epidetection of coherent signals usually selects only for coherent scattering from subwavelength objects.

Troubleshooting and Practical Limitations

Aligning a temporal focusing setup is relatively simple and forgiving of small alignment errors. Care should be taken to align the grating perpendicular to the optical axis of the telescope because the temporal focal plane is parallel to the grating surface. Care should also be taken when implementing temporal focusing with very short laser sources (<20 fsec). In this case, higher-order dispersion (i.e., third and above) should be precompensated, as it results in broadening of the axial response. Because the grating surface is illuminated at an angle, a circular laser beam profile results in an elliptic illumination pattern on the grating. Hence, a cylindrical telescope (before the temporal focusing setup) should be used to generate circular illumination patterns in the sample plane. Generally, the axial response should be equivalent to that of line-scanning multiphoton microscopy for wide-field temporal focusing and to that of point scanning for line-scanning temporal focusing. A broader axial response is usually accompanied by asymmetry and is caused by incorrect positioning of the grating along the telescope axis or is caused by an axial shift between the spatial focus and the temporal focus for line-scanning temporal focusing microscopy.

Other Applications of Temporal Focusing

Temporal focusing has recently been used in several novel imaging situations. One is rapid axial scanning. A shift in the axial position of the temporal focus corresponds to the introduction of GVD to the excitation pulse. In a recent experiment, an acousto-optic deflector was used to control the GVD, inducing a shift of the axial position of the temporal focal plane within 10 μsec (Rui et al. 2009), which is orders of magnitude faster than standard mechanical scanning devices such as direct current motors and piezoelectric crystals. Using the GVD dependence of the focal position, axial scanning was recently shown also through a fiber delivery system (Durst et al. 2006).

Selective photoactivation in a single plane has also been used to perform 3D subdiffraction-limited imaging via photoactivated light microscopy (PALM). The standard geometry for PALM limits the photoactivation volume using TIRF (total internal reflection fluorescence) excitation. This is because out-of-focus background from a thick specimen usually overwhelms the fluorescent signal from the objective focal plane. By using temporally focused two-photon photoactivation, out-of-focus background can be suppressed by orders of magnitude, enabling practical multilayer PALM (Vaziri et al. 2008).

The combination of temporal focusing with Fourier-domain spectral-pulse-shaping techniques has been used to improve the axial resolution of temporal focusing–based imaging (Oron and Silberberg 2005). This is based on a lock-in technique, relying on the fact that out-of-focus background is only weakly dependent on the pulse shape, whereas the signal from the focal plane strongly depends on it. By subtracting signals obtained with two different pulse shapes, it is possible to eliminate out-of-focus background and to achieve some narrowing of the FWHM axial response.

CONCLUSION

Temporal focusing is a versatile technique for localizing multiphoton excitation to a single plane and, as such, can replace many alternative space-multiplexing techniques in multiphoton microscopy, such as line-scanning, microlens arrays, beam-splitter arrays, and holographic beam splitters. Currently, it is the only available wide-field axially resolved multiphoton technique. Various ways to implement multiphoton imaging and photoactivation have been shown, but the possible range of applications is clearly broader, including, for example, multiphoton lithography and space-multiplexed fluorescence correlation spectroscopy. The alignment of a temporal focusing system is relatively simple, and the required components are available in practically any laboratory using ultrafast laser excitation. The technique can be practically implemented with any short-pulse (≲200 fsec) laser source.

 Cite this introduction as *Cold Spring Harb Protoc;* doi:10.1101/pdb.top085928

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Cold Spring Harb Protoc; doi: 10.1101/pdb.top085928