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Parallel and Flexible Imaging using Two-photon RESOLFT Microscopy with Spatial Light Modulator Control

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ABSTRACT

High resolution imaging in three dimension is important for biological research. RESOLFT (Reversible Saturable Optical Fluorescence Transitions) microscopy is one technique can achieve lateral super-resolution imaging. Two-photon microscopy naturally generate high resolution in the longitudinal direction with less background compared to single photon excitation. In this paper, we combine these two methods to realize three-dimensional high-resolution imaging. Spatial light modulator (SLM) is used as a flexible phase mask of the microscopy. Multiple super-resolution focuses as an array or in arbitrary positions could be generated by phase retrieval. This microscopy by SLM control could applied to parallel two-photon RESOLFT imaging or multiple spots tracking in high-resolution.

Keywords: JENLAB AWARD, Super-resolution microscopy, Two-photon microscopy, Spatial light modulator control

1. INTRODUCTION

High resolution imaging in three-dimension of large area for live specimen is important. For example, we are not only interested in imaging the whole tumor but also want to observe the metastasis of each tumor cell; we are not only want to take image of single synapse event but also want to observe the synapse circuit in a large scale[1]. Sometimes the specimen is sparse labeled, high-resolution image only in the labeled area will be more efficient[2]. Also the requirement for multiple particle tracking simultaneously of high-resolution is important to know the relationship between different particles[3]. Thus 3D high-resolution high-speed fluorescent microscopy with flexibly controlled imaging area is a good choice to facilitate the above biological research.

First, we would like to achieve lateral high-resolution, or even super-resolution. There are many methods to achieve super-resolution imaging. One type of methods is based on single molecule imaging, such as STORM (Stochastic optical reconstruction microscopy)[4] and PALM (Photoactivated localization microscopy)[5]; the other type of methods uses spatial patterned imaging, such as STED (Stimulated emission depletion microscopy)[6], RESOLFT (Reversible Saturable Optical Fluorescence Transitions)[7] and SSIM (Saturation structure illumination microscopy)[8]. RESOLFT microscopy is one super-resolution technique invented by Hell's group[7]. The technique use reversibly photoswitchable fluorophores to exploit long-live dark-state and fluorescent state. Because the comparably long "on states" and "off status" than STED microscopy, the light intensity to achieve sub-diffraction focal spot is several orders lower. In the imaging process, each scanning point is "turned on" first, and the periphery of the spot is "turned off" by a donut shape spot, then the same position is illuminated by the excitation beam and only the residue part in the center is excited and emits fluorescence. This "on-off-excitation" cycle is played in each scanning point until the whole field of view is imaged and each cycle takes about millisecond[7]. To make the RESOLFT imaging more efficient, parallel RESOLFT imaging method is published in 2013 using two incoherently superimposed orthogonal standing light waves[9]. This parallel scanning method could take a 120um by 100um super-resolution image within one second. However, this method did not consider about depth resolution, while it used sCOMS camera. So the depth resolution is the same as single photon wide-field imaging.

To improve longitudinal resolution, of course 3D super-resolution is one choice. However, the setup for 3D super-resolution imaging, especially RESOLFT method, is very complicate. On the other hand, multiphoton imaging can provide high-resolution in Z-axis. Multiphoton microscopy is widely used in biological tissue imaging, including neuroscience, embryology and oncology[10]. It enables noninvasive study of biological specimens in three dimensions with diffraction limited resolution, especially much higher longitudinal resolution compared to single photon wide-field microscopy. Multiphoton microscopy also reduces specimen photodamage as well as enhances penetration depth for thick tissue. Recently, multiphoton imaging is used to do *in vivo* imaging of subcortical structures within an intact mouse

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brain[11], as well as blood flow and neurovascular coupling in the brain[12]. Multiphoton microscopy could also combine with other techniques, which melt the advantages of both methods. For example, two-photon excitation STED microscopy improved the spatial resolution of standard two-photon microscopy by a factor of four to six times, which is used to image morphology of dendritic spines and microglial cells of brain slice[13].

In this paper, we designed a new setup based on RESOLFT microscopy. The setup contains three beams: "turn-on" light, "turn-off" light, and excitation light. "Turn-on" light is provided by a LED around 405nm wavelength. "Turn-off" light and excitation light are two-photon excitation, switching by a SLM. The SLM also controls the vortex phase for "turn-off" light, as well as system phase aberration compensation. The microscopy also can do parallel scanning by generating a phase grating on the SLM. The grating size and separation is adjustable. The parallel scanning can improve imaging speed. On the other hand, the microscopy can do specific area scanning or multiple super-resolution particle tracking by generating multiple super-resolution spots in arbitrary positions. The method may be used to imaging single synapse event or tracking several synapses events simultaneously.

2. THEORY AND METHOD

Parallel two-photon RESOLFT microscopy setup

The experiment setup is shown in figure 1. The specimen is illuminated by the UV "turn-on" light first. Then, the periphery area of the spot is "turned-off" by the donut shape two-photon light. Finally, the residue center area which is still in "on-state" is excited by the two-photon Gaussian spot and emit fluorescence. The AOM and TTL trigger for LED control "turn-on" and "turn-off" time sequence, synchronized by a NI card (National Instrument, USB-6009). The SLM controls "turn-off" and excitation sequence by LabVIEW. For each spot, the camera takes a wide-field image. Signal was extracted from the image as one-pixel intensity of the final reconstructed image.

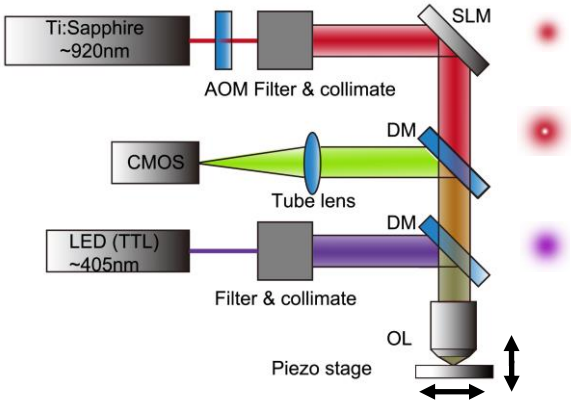


Figure 1. The experiment setup for parallel two-photon RESOLFT microscopy. Mode-locked Ti:Sapphire laser (Spectra-physics, Tsunami) provides the source for "turn-off" light and excitation light. AOM (acoustic optical modulator, ISOMET) synchronized with LED (Thorlabs, M405L2, wavelength ~405nm) to control "turn-on" and "turn-off" time sequence. SLM (Spatial light modulator, HOLOEYE PLUTO) provides the phase control of the system. Other main components: CMOS (Point Grey, Flea3 USB), OL (objective lens, Zeiss A-Plan, x100, NA 1.25), DM: dichroic mirror, piezo stage (Queensgate, NPS-XY-100A).

Specimen and fluorescent labeling

We use *live* HEK (Human Embryonic Kidney) cell as specimen. The reversibly photoswitchable fluorophore used in the experiment is Dronpa-M159T[14] which is used in RESOLFT microscopy before. The tubulin of HEK cell is labeled by Dronpa-M159T by genetically transfection. Dronpa-M159T is a robust reversibly photoswitchable fluorophore, with comparably high quantum yield (Φ_{FL}=0.23) and molar absorption coefficient (61732 $M^{-1}cm^{-1}$)[15]. Dronpa-M159T has much shorter switch-off half time and switch-on half time compared to the other Dronpa series under the same illumination intensity. The absorption spectrum is maximum around 489nm in "on-state" and around 405nm in "off-state", and the maximum of emission spectrum is about 515nm[15].

Phase retrieval

The phase retrieval of arbitrary position multi-focus uses the classic Gerchberg-Saxton iterative algorithm modified by Fienup[16]. The principle of the iterative Fourier transform (FT) algorithm is figure 2. For the signal changing from kth loop to k+1, the iterative equation is

$$A_{k+1} = A_k' + \beta \Delta A_{driving,k}, \Delta A_{driving,k} = |A_T| \left\{ 2\exp\left[i \cdot \arg\left(A_k' \right) \right] - \exp\left[i \cdot \arg\left(A_k \right) \right] \right\} - A_k \tag{1}$$

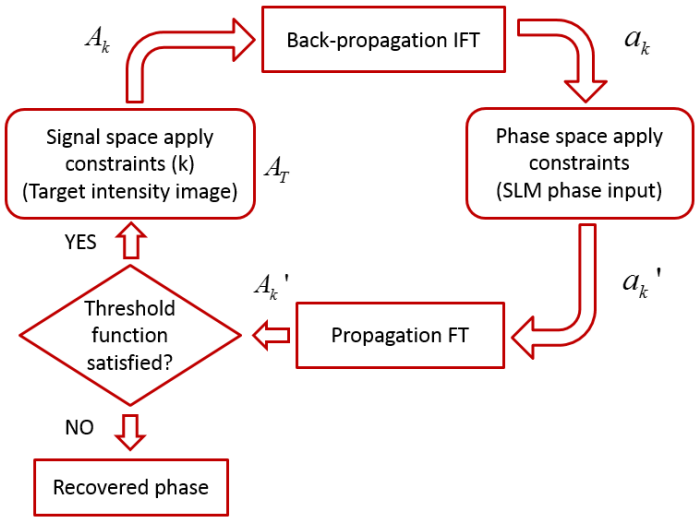


Figure 2. The principle of the iterative Fourier transform algorithm.[16]

where β is a free parameter, usually chosen close to one. This method has high efficiency to converge. When we already know the aimed position of multiple focuses A_T, the phase on the back focal plane is calculated iteratively until converge. Add this retrieved phase to the vortex 0-2π phase can generate donut spots in the corresponding position. Thus, it is possible to locally scanning and do super-resolution imaging in several sub-regions simultaneous. This flexible imaging method has many potential application in biological research. For example the method can be used to image sparse aims in the specimen, which is more efficient than scanning the whole field of view. For small area scanning, it is possible to do live imaging for several sub-regions, such as do multiple super-resolution tracking for several connected synapse events.

System aberration correction

The system aberration is corrected by adaptive optics method[17]. The intensity of the focal spot is measured, and then the sharpness and brightness of the spot are calculated. The image brightness B is the sum of the pixel values of the image, and the sharpness S is defined as the second moment of the image Fourier transform as

$$S = \sum_{n,m} \mu_{n,m} \hat{I}_{n,m} \left(n'^2 + m'^2 \right) / \sum_{n,m} \hat{I}_{n,m} \tag{2}$$

where $\hat{I}_{n,m}$ is the Fourier transform of the image, n' and m' are the coordinate relative to the center of the image. $\mu_{n,m}$ is a low-pass filter with the radius w. The combined metric of brightness and sharpness is defined as

$$M = S + \sigma \beta B \left[\frac{1}{1 + e^{-k(S - S_T)}} \right] \tag{3}$$

Sharpness is the main concern unless the sharpness is above the threshold S_T, which is normally chosen as the 90% of the maximum sharpness. σ is equal to +1 or -1 decided by the M is the maximum or minimum value. β and k are the experiment value to balance the contribution of sharpness and brightness.

During the aberration measurement, add different degree of aberration to the original phase input. For example, when the input phase is vortex 0-2π ϕ_{vor}, the testing phase is $\phi = \phi_{vor} + bZ_i, b \in [-1,1]$, Z_i is ith Zernike mode representing aberration. Adding different ϕ to the SLM and calculate the metric M of each image, and parameter b for the highest M is the compensate factor. Different aberration (incoherent) could be corrected simultaneously.

3. RESULT AND DISCUSSION

First, we measured the time sequence and PSF (point spread function) of the two-photon RESOLFT microscopy. The results are shown in figure 3. Because the "turn-on" light is from LED, the PSF is much larger than the other two beams. The three images are taken by the CMOS camera.

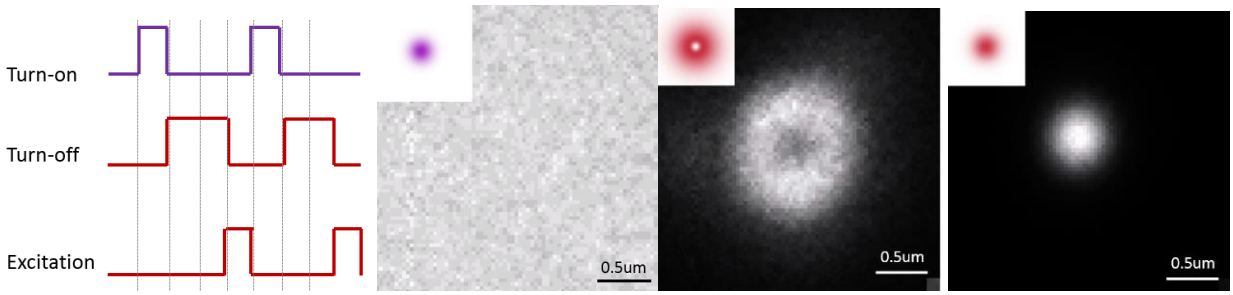


Figure 3. The time sequence of three light of RESOLFT microscopy section: "turn-on" light (UV light), "turn-off" donut shape light, and excitation Gaussian light.

Second, figure 4 shows the SLM controlled spots positions. We can generate regular spot matrix (figure 4(a) shows a 5x5 spot matrix as an example), and change the spatial frequency of the grating by simply zoom in and zoom out the phase image on the SLM. Figure 4(b) shows the result of generating arbitrary spots positions. In this process, we randomly generate the coordinate of the aimed spots, and do the phase retrieval calculation to recover the phase. Noticed that the intensity of each spot is not perfectly uniform, this could be improved by more iterative loops, or set the target rule as the standard deviation of intensity is smaller than a certain threshold. According to the same algorithm, but adding an extra vortex 0-2π phase to the recovered phase, this is equal to convolve a donut shape PSF to each Airy spot. The results are shown in figure 5.

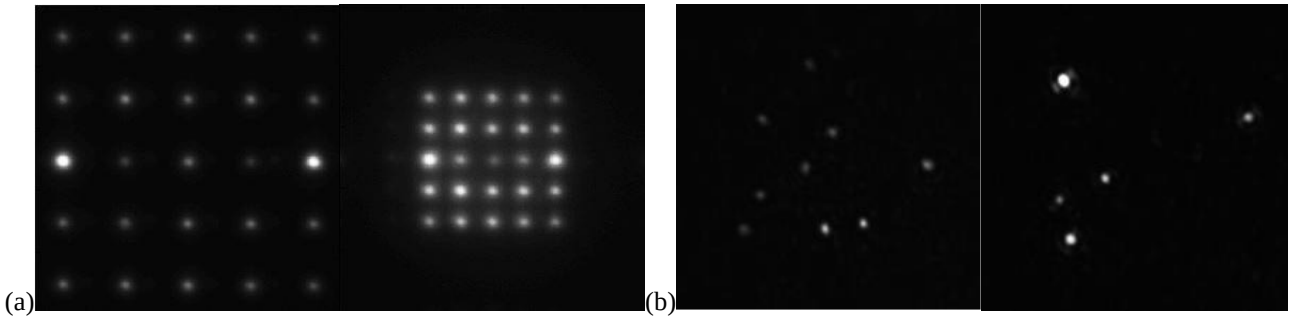
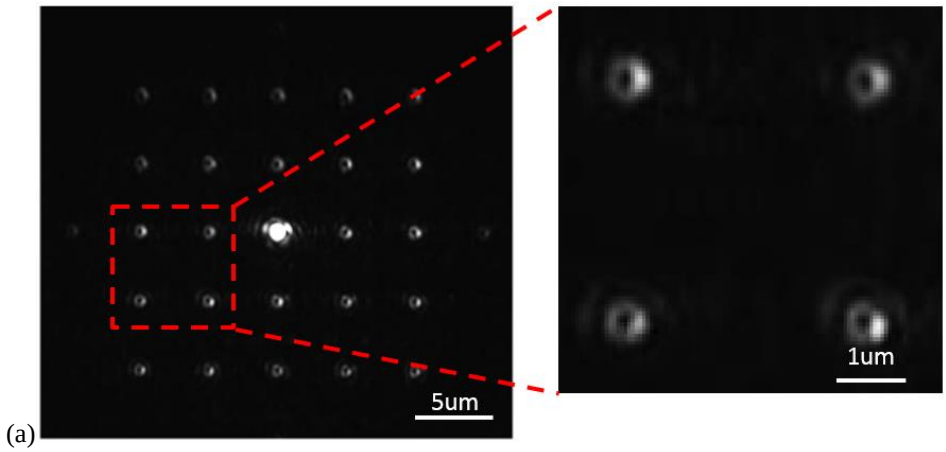
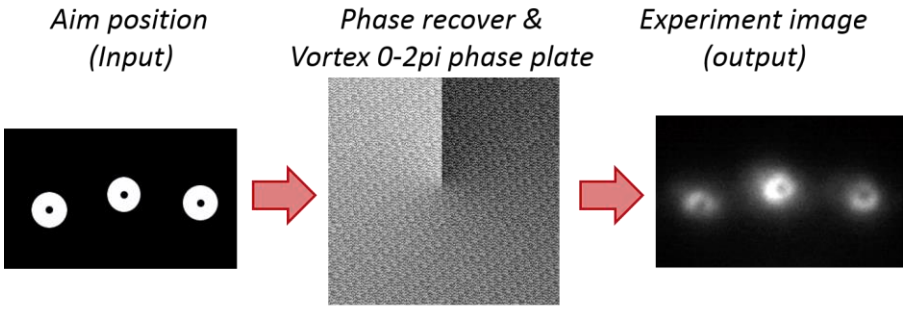


Figure 4. SLM controls spots position. (a) spots matrix for parallel imaging; (b) arbitrary positions for multiple particle tracking.



(a)



(b)

Figure 5. SLM controls spots position. (a) The spots matrix for parallel super-resolution imaging; (b) flexible position of donut spots, for multiple super-resolution particle tracking

We also applied this two-photon RESOLFT microscopy system to biological imaging for testing. The specimen is HEK cell tubulin labeled by Dronpa-M159T fluorophore. Figure 6 shows the two-photon point scanning image of this specimen. The right side image is the sub-region of left image with higher resolution scanning (200nm/pix). From the image, we can tell the detail of tubulin in high-resolution, which as an example demonstrates the image quality of our experiment system.

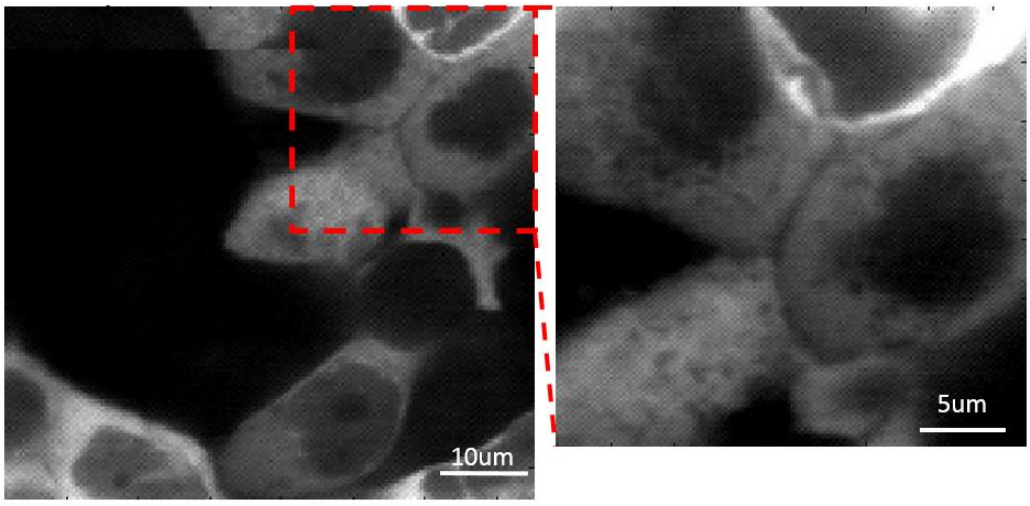


Figure 6. HEK cell specimen two-photon imaging. Left: 60x60um field of view. Right: 30x30 um field of view of sub-region re-scanning with 200um/pix step length.

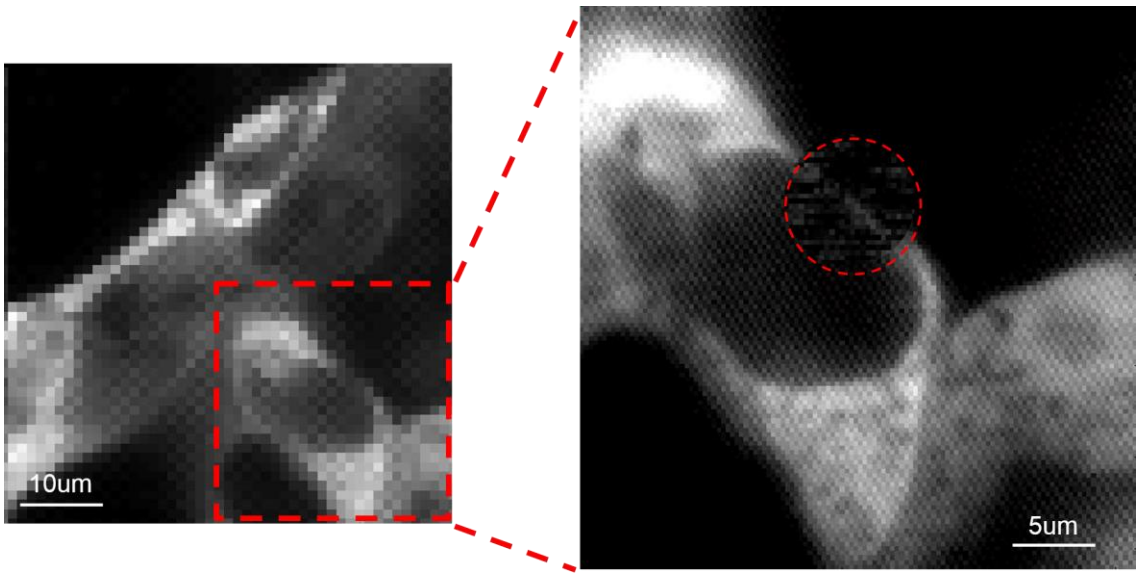


Figure 7. HEK cell specimen two-photon imaging RESOLFT super-resolution imaging. Left: 50x50um 1um rough scanning image; Right: 25x25 um field of view of sub-region re-scanning with 200um/pix step length; red-circle region: Two-photon RESOLFT image of the same position sub-region in the back image.

Figure 7 shows the resolution increasing from the 1um resolution to 200um resolution, and finally to sub-diffraction resolution. Using two-photon RESOLFT microscopy we are able to distinguish more precision details of cellular or sub-cellular level. This potentially will help us to explore the correlated biological field further.

4. CONCLUSION

We designed a two-photon RESOLFT microscopy with SLM control, which can apply to high speed 3D high-resolution imaging by parallel scanning, as well as high-resolution multiple particle simultaneously tracking by flexible position scanning. The system can achieve less aberration imaging via phase compensation. We showed the algorithm and experiment results of these unique characteristics in the paper. Iterative phase retrieval algorithm is used to calculate the phase mask on SLM for flexible position imaging. Phase compensation using adaptive optics is applied to reduce aberration. As for the experiment result, we showed the PSF and time sequence of the three beams in the RESOLFT microscopy section. For parallel imaging or flexible imaging, both Gaussian spots (excitation beam) and donut spots ("turn-off" beam) experiment images are shown. At the last, the two-photon images of the cell specimen labeled by reversibly photoswitchable fluorophore are demonstrated.

At the same time, the design could be further promoted by higher speed scanning, such as using resonant mirror instead of piezo stage. This will improve the image system efficiency, making the live imaging possible. The other drawback is the absolute intensity of super-resolution image is low, because the fluorescence was turned off in the surrounding area and only limited area emits fluorescence. Thus, the signal to noise ratio reduced compared to the two-photon images. sCMOS camera or other more sensitive detector with less background noise could improve the image quality further. After these modification, the large field of view, high-speed, high-resolution microscopy could be realized. This microscopy is potentially used for imaging large area of specimen with 3D high-resolution in detail, such as single synapse event as well as the synapse circuit in the brain slice *in vivo*.

REFERENCES

[1] Guo, S. M., Veneziano, R., McConnell, R. E., Agasti, S., Gordonov, S., Kulesa, T., ... & Bathe, M. Quantitative Multiplexed Super-Resolution Neuronal Synapse Imaging using DNA-Paint. Biophysical Journal, 108(2), 477a (2015).

[2] Kwon, H. S., Nam, Y. S., Wiktor-Brown, D. M., Engelward, B. P., & So, P. T. Quantitative morphometric measurements using site selective image cytometry of intact tissue. Journal of The Royal Society Interface, 6(Suppl 1), S45-S57, (2009).
[3] Adu-Gyamfi, E., Digman, M. A., Gratton, E., & Stahelin, R. V. Single-particle tracking demonstrates that actin coordinates the movement of the Ebola virus matrix protein. Biophysical journal, 103(9), L41-L43 (2012).
[4] Rust, M. J., Bates, M., & Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nature methods, 3(10), 793-796. (2006).
[5] Betzig, E., Patterson, G. H., Sougrat, R., Lindwasser, O. W., Olenych, S., Bonifacino, J. S., ... & Hess, H. F. Imaging intracellular fluorescent proteins at nanometer resolution. Science, 313(5793), 1642-1645. (2006).
[6] Hell, S. W., & Wichmann, J. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. Optics letters, 19(11), 780-782. (1994).
[7] Testa, I., Urban, N. T., Jakobs, S., Eggeling, C., Willig, K. I., & Hell, S. W. Nanoscopy of living brain slices with low light levels. Neuron, 75(6), 992-1000. (2012).
[8] Gustafsson, M. G. Nonlinear structured-illumination microscopy: wide-field fluorescence imaging with theoretically unlimited resolution. Proceedings of the National Academy of Sciences of the United States of America, 102(37), 13081-13086. (2005).
[9] Chmyrov, A., Keller, J., Grotjohann, T., Ratz, M., d'Este, E., Jakobs, S., ... & Hell, S. W. Nanoscopy with more than 100,000'doughnuts'. Nature methods, 10(8), 737-740. (2013).
[10] So, P. T., Dong, C. Y., Masters, B. R., & Berland, K. M. Two-photon excitation fluorescence microscopy. Annual review of biomedical engineering, 2(1), 399-429. (2000).
[11] Horton, N. G., Wang, K., Kobat, D., Clark, C. G., Wise, F. W., Schaffer, C. B., & Xu, C. In vivo three-photon microscopy of subcortical structures within an intact mouse brain. Nature photonics, 7(3), 205-209. (2013).
[12] Shih, A. Y., Driscoll, J. D., Drew, P. J., Nishimura, N., Schaffer, C. B., & Kleinfeld, D. Two-photon microscopy as a tool to study blood flow and neurovascular coupling in the rodent brain. Journal of Cerebral Blood Flow & Metabolism, 32(7), 1277-1309. (2012).
[13] Bethge, P., Chéreau, R., Avignone, E., Marsicano, G., & Nägerl, U. V. Two-photon excitation STED microscopy in two colors in acute brain slices. Biophysical journal, 104(4), 778-785. (2013).
[14] Lavoie-Cardinal, F., Jensen, N. A., Westphal, V., Stiel, A. C., Chmyrov, A., Bierwagen, J., ... & Hell, S. W. Two-Color RESOLFT Nanoscopy with Green and Red Fluorescent Photochromic Proteins. ChemPhysChem, 15(4), 655-663. (2014).
[15] Egner, A., Geisler, C., Von Middendorff, C., Bock, H., Wenzel, D., Medda, R., ... & Hell, S. W. Fluorescence nanoscopy in whole cells by asynchronous localization of photoswitching emitters. Biophysical journal, 93(9), 3285-3290. (2007).
[16] Fienup, J. R. Phase retrieval algorithms: a comparison. Applied optics, 21(15), 2758-2769. (1982).
[17] Gould, T. J., Burke, D., Bewersdorf, J., & Booth, M. J. Adaptive optics enables 3D STED microscopy in aberrating specimens. Optics express, 20(19), 20998-21009. (2012).