doi:10.1017/S1431927611000900
Microsc. Microanal. 17 (Suppl 2), 2011

Multi-Color Interferometric Photo-Activation Localization Microscopy with Extended Axial Range

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Interferometric Photo-Activation Localization Microscope [1] can measure the 3D positions of individual molecules with accuracy of 10-20 nm even for relatively dim fluorescent proteins. This facilitates high resolution optical imaging with expressed genetically encoded fluorescent-tagged proteins providing high specificity, short linker length, and high labeling density.

The original iPALM method allows for both high lateral *x-, y-* localization and high vertical *z*-localization of a fluorescent particle over a vertical range of single interferometric fringe ~250nm. Beyond that range, the high precision can be maintained with the next interference fringe; however one can't discriminate between adjacent interference fringes. In order to overcome this z-coordinate ambiguity we replaced the original turning 22.5º mirrors in iPALM setup with mildly hyperbolic mirrors that added a saddle shaped phase shift across the pupil plane. As a result, the Point-Spread Functions (PSF's) of images of point sources became elliptical, and this ellipticity varies with axial coordinate [2]. The X-Y ellipticity of astigmatic PSF's can be defined as:

$$\varepsilon = \frac{\sigma_x - \sigma_y}{\sigma_x + \sigma_y} \quad (1),$$

where σ_x and σ_y are Gaussian widths along *x*- and *y*- axes. The measured dependence of X-Y ellipticity on z-coordinate can be used to extract *z*-coordinate. This method has much lower accuracy than the iPALM, but is not limited to a single interferometric fringe and therefore can be used to determine the fringe order, allowing for accurate determination of *z*-coordinate over a wider range.

This is performed in a following way. During the iPALM calibration, the dependence of X-Y ellipticity on sample *z*-coordinate is recorded along with the standard iPALM interferometric calibration. The polynomial fitting is performed according to the following formula:

$$Z_{sample} = m_1 + m_2 \bullet \varepsilon + m_2 \bullet \varepsilon^3 \quad (2).$$

Then, during the iPALM measurements we extract two values Z_{interf}, obtained interferometric data as described in [1], and $Z_\varepsilon = m_1 + m_2 \bullet \varepsilon + m_2 \bullet \varepsilon^3$, obtained using the calibrated dependence of X-Y ellipticity on sample position and measured value of ε for each fluorescent event. Then the actual *z* – coordinate is calculated using the formula:

$$Z = Z_{\text{interf}} + Z_{\text{interf.fringe}} \cdot Round\left(\frac{Z_\varepsilon - Z_{\text{interf}}}{Z_{\text{interf.fringe}}}\right) \quad (3),$$

where, the constant $Z_{interf.fringe}$ is a single interferometric fringe ~250 nm.

The procedure works well over the range of over ~750 nm, which is close to the depth of field of the high NA objective used in the experiment. There is a small fraction of molecules for which the interferometric fringe is determined incorrectly, the total fraction of these ghosts, or Error Ratio is usually below 5%.

Another limitation of original iPALM technique was its single-color design, which limits the scope of the problems that can be addressed. Ideally one would want to be able to image multiple proteins labeled with different fluorescent labels within a single sample. Multi-color super-resolution imaging requires a way to differentiate fluorescent labels. This can be done by constructing labels with different excitation spectra and identical emission spectra [3], or using fluorescent labels with different emission spectra [4]. In the latter case iPALM images in different color channels with different fluorescent wavelengths are typically not aligned "as acquired", images may be shifted and tilted due to different filter sets used in acquisition and slight magnification difference of optics at different wavelengths. Thus the images in different channels need to be aligned, and the alignment accuracy needs to be evaluated, since co-localization of fluorescent labels in 2 channels is determined by the following equation [5]:

$$\sigma = \sqrt{\sigma_{loc1}^2 + \sigma_{loc2}^2 + \sigma_{reg}^2} \quad (4),$$

where, σ_{loc1} and σ_{loc2} are the localization accuracies in each color channel, and σ_{reg} the accuracy with which positions between 2 channels can be registered.

Images collected in 2 color channels should be related by a similarity transformation involving shift, rotation, and slightly different magnifications. In order to determine the transformation parameters for such transformation, we need to register certain near point source objects with fluorescence in both color channels. Au nanoparticles, such as nano-balls or nano-rods can be used, since they have surface-plasmon-resonance (SPR) enhanced photoluminecscence with wide spectra in visible region [6,7]. Bare Au nano-rods are particularly attractive since they have two SPR modes and their spectra may be custom-tailored to cover the spectra of fluorescent labels used in the experiments [7].

We had evaluated the registration accuracy σ_{reg} with both 80 nm Au spherical nanoparticles and 25 nm x 35 nm nanorods for 520 nm and 590 nm channels. In each case we imaged these nanoparticles and determined their coordinates in both channels. We then extracted the similarity transformation coefficients using linear regression. After the transformation was performed, we calculated the differences between the new *x*- *y*- and *z*- coordinates of each nanoparticle in 2 channels, which allowed us to estimate the registration accuracy σ_{reg}. In most cases it is below 10 nm. We are currently applying the multi-color extended z-range iPALM to a number of biological problems, we will show the results of these studies.

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https://doi.org/10.1017/S1431927611000900 Published online by Cambridge University Press