OPTOSPLIT II

Application Note

OptoSplit II, an image splitter optimised for 2D and 3D super-resolution microscopy:

Resolution in far-field light microscopy is limited to ~200–250 nm, hindering our ability to 'see' the structure of various subcellular features (e.g. the nuclear pore complex). Super-resolution microscopy, including the single molecule localisation methods (**SMLM**), e.g. **PALM**, and **STORM**, can achieve up to a 10-fold improvement in lateral resolution, largely dependent upon the fluorescent probe used. For **SMLM** techniques, it is important to limit the number of fluorescent molecules that are 'active' (i.e. emitting photons) at any one time so that each molecule can be distinguished individually.

In 'direct' **STORM** (or **dSTORM**), this is achieved by encouraging the fluorescent dye molecules to 'blink' (i.e. to switch on and off), a phenomenon that requires a particular set of experimental conditions (e.g. the composition of the imaging buffer, etc). Furthermore, the precision with which a single molecule can be localised is dependent (at least in part) upon the number of photons emitted by the molecule; the greater the number of photons detected, the greater the localisation precision.

Simultaneous super-resolution imaging of two fluorophores can be achieved using an image splitter such as the **OptoSplit II**, manufactured by **Cairn Research**. Here, a dichroic mirror separates the emitted light into two distinct spectral bands, aided by the use of an appropriate bandpass emission filter for each channel. **The OptoSplit II** is designed to maximise photon throughput to the detector (most often a **CMOS** or **EMCCD** camera), which is a key requirement for this application, and is supplied with bespoke, multi-element relay lenses and 'ultraflat' dielectrically-coated mirrors for this purpose.

Dr Ruisheng Lin is a postdoctoral research associate in the laboratory of Professor Christian Soeller at the University of Exeter. One of the main focus areas of this group is the relationship between cellular architecture at the nanometre scale and the contractile function of cardiac muscle. Utilising a broad range of fluorescence-based microscopy techniques, they are furthering our understanding of the molecular machinery involved in the beating of cardiac muscle. Members of this laboratory regularly use the **OptoSplit II** for dual colour **dSTORM** and '**DNA** Points Accumulation for Imaging in Nanoscale Topography' (**DNA PAINT**) imaging[1].

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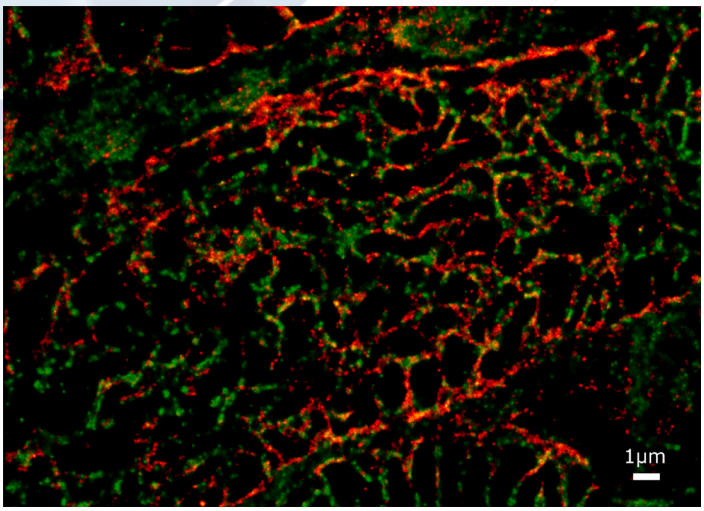

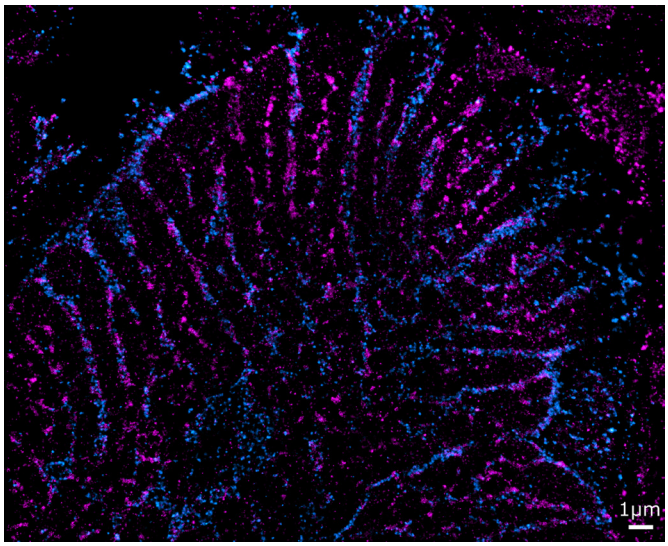

Figure 1

Figure 2

Figure 1 - A dual-colour **dSTORM** image of pig cardiac tissue is shown, labelled with Alexa Fluor 647 and Alexa Fluor 700 in an aqueous, gluc ox, **MEA** buffer. The targets are **SERCA2 ATPase** (red, A700) and **RyR2** (green, A647). Sample prepared and imaged by Dr. Alex Clowsley (University of Exeter).

Figure 2 - A dual-colour **DNA PAINT** image of pig cardiac tissue showing the localisation of ryanodine receptors (magenta) relative to the transverse tubular system ('t-system', blue). In comparison to the dye blinking technique of **dSTORM**, **DNA PAINT**, which relies upon the hybridisation of single-stranded, fluorescently labelled **DNA** oligonucleotides, offers the advantage of being able to use very bright fluorescent dyes in combination with imaging buffers designed to optimise their photon output, thereby enabling a localisation precision of <10nm. [2, 3] Sample prepared and imaged by Dr. Alex Clowsley and Anna Meletiou (University of Exeter).

Extending the resolution improvement from **2D** to **3D** presents additional challenges, but there are many elegant approaches that can be adopted to determine the axial (or 'z') position of a single molecule. Using 'Interferometric' **PALM** (**iPALM**), for example, an axial resolution of 10–20 nm is achievable[4]; however, the associated instrumentation is complex and may prove difficult to assemble for the non-specialist. Alternatively, simpler approaches such as astigmatism, which can alter the shape of a molecule's point spread function (**PSF**), can be adopted. Here the **PSF** is 'engineered' in such a manner that it can encode **3D** information, whereby the axial position of an individual molecule is determined by the shape of its projected image on a camera. Another approach, referred to as **3D** biplane microscopy[5], involves imaging two different focal planes within the same sample simultaneously, enabling the axial position of an individual molecule to be derived from the two images. An image splitter device such as the **OptoSplit II** can also be used for this purpose.

[1] Jungmann, R. et al. (2017), Super-resolution microscopy with DNA-PAINT, Nature Protocols **12**: 1198-1228
[2] Jungmann, R. et al. (2014), Multiplexed 3D cellular super-resolution imaging with DNA-PAINT and Exchange-PAINT, Nature Methods **11**: 313-318
[3] Jayasinghe, I. et al. (2018), True molecular scale visualisation of variable clustering properties of ryanodine receptors, Cell Reports **22**: 557-567
[4] Shtengel, G. et al. (2009), Interferometric fluorescent super-resolution microscopy resolves 3D cellular ultrastructure, PNAS **106**: 3125-3130
[5] Juette, M. et al. (2008), Three-dimensional sub-100nm resolution fluorescence microscopy of thick samples, Nature Methods **6**: 527-529

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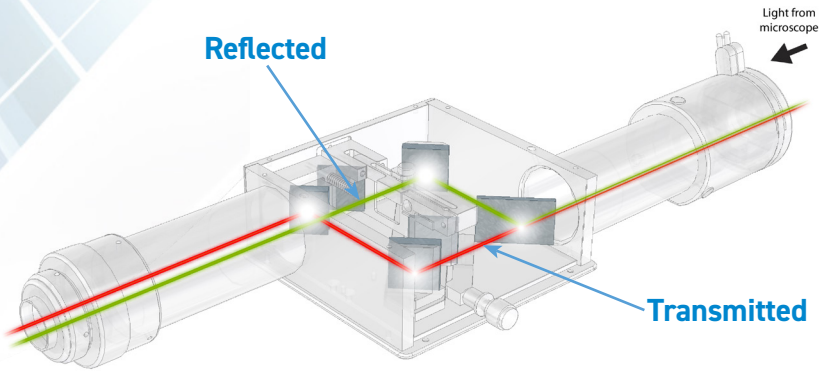
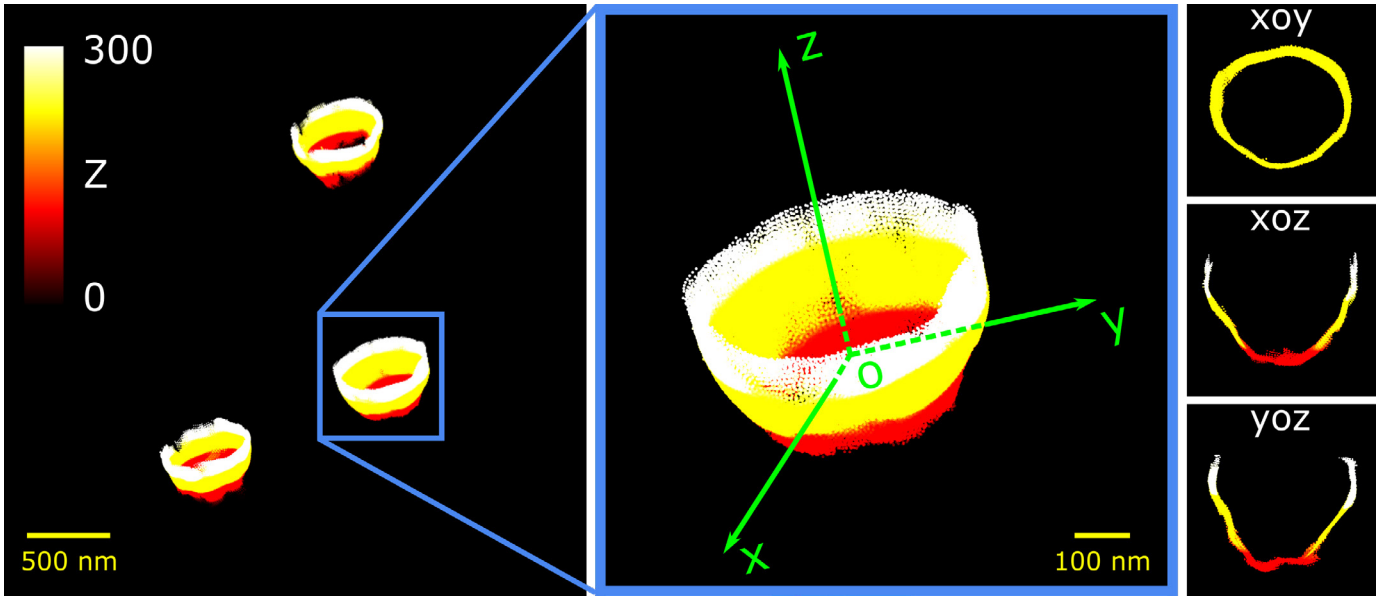


Figure 3

In the 'biplane' configuration, emitted light passes through a 50:50 non-polarising beam splitter, which effectively splits the light into two paths. As shown in **Figure 3** these are labelled as '**transmitted**' and '**reflected**', with each arriving and producing a different image on the sensor of the camera attached to the **OptoSplit**. By inserting a lens into either the transmitted or reflected path it is possible to ensure that each path 'sees' a different focal plane within the sample. An advantage of the biplane approach is speed, because you can capture images of two different focal planes simultaneously without waiting for a scanning device (such as a piezo z stage) to move to its next position. As this approach divides the emitted fluorescence signal in two, the design of the image splitter as well as the quality of the optical components used is key to ensure that none of the emitted photons are wasted.



Images of beads after 3D drift correction, clearly showing the expected 'bowl' structure because the illumination thickness is approximately half the bead diameter

An enlarged view of a single bead, as well as several different cross-sections of this bead are shown, with the orientation of each cross-section provided. A best-fit representation of the data points is shown for each bead, where each data point represents the position of fluorescently-labelled DNA oligonucleotide. Each colour of the LUT corresponds to a different axial position.

Figure 4

Figure 4 - 500nm polystyrene beads imaged using DNA PAINT. Sample prepared by Tobias Lutz and imaged by Dr. Ruisheng Lin (University of Exeter)

In addition to dual colour imaging, Dr. Ruisheng Lin (University of Exeter) also uses the **OptoSplit II** for biplane-type **3D** super-resolution microscopy. **Figure 4** shows a series of polystyrene beads of 500 nm diameter, imaged using **DNA PAINT**. The beads have **DNA** 'docking' strands attached to their surface and complementary **DNA** '**imager**' strands, each of which is coupled to a fluorescent dye, are added to the sample containing the beads. The '**imager**' strands transiently bind to the '**docking**' strands, producing '**blinks**' in the image which can then be localised. 'Total Internal Reflection Fluorescence' (**TIRF**) illumination was used in this instance and so the thickness of excitation was limited to several hundred nanometres. The axial range covered in this biplane experiment was 0–300 nm, covering just over half the diameter of the beads.

Conclusion

The **OptoSplit II** has been specifically designed with super-resolution microscopy in mind, offering an end user the following:

- **Maximised throughput**: the optimised design, including AR-coated achromatic triplet lenses and dielectrically coated mirrors maximises the transmission of emitted photons from microscope to camera.

- **Distortion-free images**: the two internal optical pathways of the **OptoSplit** are symmetrical and of equal length, which is essential for ensuring that the images of the two channels can be precisely overlaid. Furthermore, the AR-coated achromatic lenses are designed to minimise optical aberrations and thereby preserve the shape of the diffraction-limited PSF. In addition, the substrate on to which the dielectric coating of each internal mirror is deposited is such that surface flatness is optimal for demanding applications such as super-resolution microscopy. The filter cube contained within the **OptoSplit** can also accommodate '**UltraFlat**' dichroics of 3 mm thickness from Chroma (offering a surface flatness of <0.25 waves/inch Peak–Valley). All these design considerations serve to maximise image quality and uniformity, as well as signal-to-noise ratio.

- **Stability**: the robust mechanical design and alignment controls provide a stable platform, ideal for long-term imaging. This is important because single molecule localisation-based techniques often require the acquisition of many thousands of imaging frames.

- **User friendly, flexible design**: the alignment controls are simple and intuitive, easing the process of optimisation before starting an experiment. It is also possible to change the magnification of the **OptoSplit**, helping to match the pixel size of the camera to the size of the **PSF**, thereby aiding the fitting process to determine the position of a single molecule.

- **3D imaging**: the **OptoSplit II** supports several different approaches for determining the axial (or z) position of a single molecule, including biplane imaging, and **PSF** engineering methods such as astigmatism. *Should you wish to utilise a phase mask for shaping the PSF, please speak to us about accessing the pupil plane.*

The **OptoSplit II** is part of a family of image splitter products that also includes the **OptoSplit III** and **MultiSplit**, which are three-way and four-way emission image splitters, respectively. Apart from super-resolution microscopy, these devices can be used for a broad range of other imaging applications including polarisation studies (**anisotropy**), ratiometric calcium imaging, simultaneous multi-focal plane imaging and more. For the latest application notes relating to other products within **Cairn Research**'s expanding product portfolio, please visit our website:

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