Aplo Scope

Multicolor target labelling at cell biology research

Introduction

Cellular response to stresses and stimuli often requires coordination between multiple organelles and signalling hubs. For many years the mitochondria was mostly famous for being the powerhouse of the cell, and while that is true, more recent discoveries suggest that the mitochondria also plays many other roles, including in global metabolic homeostasis and in response to infections[1-3].

Numerous studies describe a bidirectional crosstalk between the mitochondria and cellular innate immune pathways, primarily type 1 interferon and autophagy. For instance, one of the key players in the response to RNA viruses is the adaptor protein MAVS, located at the mitochondrial membrane and transducing signals which start there and culminate in transcription of interferon and interferon-stimulated genes[1,4]. Equally, a number of immune events also require the involvement of the mitochondria, for example, the production of reactive oxygen species (ROS) and certain types of programmed cell death. Interestingly, MAVS was also suggested to be directly linked with the autophagy mediator LC3, affecting both mitochondrial homeostasis and Rig-I Like Receptor (RLR)-mediated autophagy[2]. Likewise, autophagy itself is a nuanced process that requires the coordination of multiple cellular components, from membrane receptors to gene expression to lysosomes. When "branching out" from canonical autophagy, one has to consider overlapping pathways like ER-phagy and mitophagy, further highlighting the crosstalk of lysosomes and mitochondria with other organelles, including the secretory pathway[5-7].

Summary

ONI's Aplo Scope and accompanying software tools, including cloud-based CODI, offer a versatile platform to simultanously detect, quantify, and report multiple cellular targets. The combination of diffraction-limited imaging and SMLM provides highly detailed description of structural elements at the correct cellular context in a timely manner.

Taken together, studying one organelle or cellular pathway is inevitably leading to studying additional links in the complex cellular network. It is impossible to understand the mitochondria, for example, without addressing how its fragments are moving along the cytoskeleton or how it communicates with the secretory organelles. This sort of research requires the study of multiple cellular targets under different conditions and stimuli. In this App note we provide a proof of principle for immunofluorescence-based examination of five different cellular targets in the same sample: Some are imaged in super-resolution to provide a detailed structural characterization, while other are imaged in diffraction-limited conditions and are used to give an accurate cellular context.

Challenge

Immunofluorescence labelling of more than four targets is considered very challenging since standard microscopes have four lasers, corresponding to four distinct "colors". Furthermore, matching 3 or 4 primary antibodies with secondary antibodies of different species is even more challenging. For dSTORM experiments, standard fluorescently labeled antibodies might not suffice, as the fluorophores have to be able to blink, which also requires specific buffers and imaging conditions. These restrictions make multiplex immunofluorescence-based assays very challenging, especially when including SMLM techniques like dSTORM or DNA-PAINT.

Methods

Human U2OS cells were plated into Ibidi 6-well µ-plates and placed in culture for 24h. Cells were then washed with PBS and fixed with ONI Discovery Kit™ for dSTORM in Cells Fixative B, paraformaldehyde (PFA) supplemented with glutaraldehyde for strong fixation. Cells were subsequently washed, quenched, permeabilized, and blocked using the relevant reagents provided with the Discovery Kit: dSTORM in Cells. Target probing was performed using directly conjugated primary antibodies in the following arrangement: anti-Histone H3 (acetyl K27)-CF®405S, anti-Alpha Tubulin-CF®405L, anti-LAMP1-Atto 488, anti-Clathrin Heavy Chain-CF®583R, anti-TOMM20-AZDye™ 647. Staining was performed in PBS + 5% bovine serum albumin, with all antibodies at a final concentration of 10 µg/ml, for 2 h at room temperature before samples were washed 3 times with PBS and fixed again with 4% PFA. Before imaging, samples were covered with freshly prepared ONI's dSTORM Imaging Buffer. All imaging was performed on the Aplo Scope. Diffraction limited images were taken as 10 frames/channel at 100 ms exposure, whilst dSTORM images were collected as 20,000 frames/channel at 30 ms exposure. Imaging was performed using an illumination angle of 52-54°, ensuring robust total internal reflection fluorescence (TIRF).

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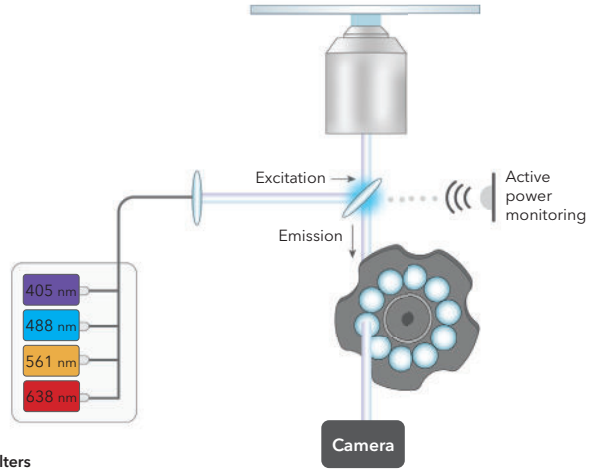
Results

The optical configuration of Aplo Scope is designed to facilitate a diverse range of experimental set-ups flexibly and in a manner compatible with the vast majority of common imaging fluorophores. The system incorporates four laser lines at 405 nm, 488 nm, 561 nm, and 638 nm, all capable of stable operation at low powers (< 1 mW) ideal for live or fixed diffraction-limited imaging and high powers (> 100 mW) for SMLM applications. This is combined with five filter options. Overall this enables a varied range of laser-filter combinations that can robustly separate a wide range of fluorophores (Fig. 1).

Figure 1 | Schematic of Aplo Scope optical path



Aplo Scope filters

1. 445/30
2. 527/49
3. 595/44
4. 673/35
5. 760/110
6. 519/35 | 605/35
7. 519/35 | 673/35
8. 605/35 | 673/35
9. 446/16 | 515/25 | 597/25 | 673/35

Here, the multicolor capabilities of Aplo Scope were exemplified using samples prepared from human U2OS cells and probed with a five-dye antibody panel. All were commercially available fluorophores directly conjugated to primary antibodies, and represent one small subset of the wide range of dye combinations compatible with Aplo Scope. These were: anti-Histone H3-CF®405S (nuclear), anti-Alpha Tubulin-CF®405L (cytoskeletal), anti-LAMP1-Atto 488 (lysosomal), anti-Clathrin Heavy Chain-CF®583R (plasma membrane), and anti-TOMM20-AZDyeT™ 647 (mitochondrial). The use of primary antibodies only allowed some antibody isotypes to be used for multiple targets, however a primary-secondary antibody approach would also be effective with the correct panel design. The fluorophore panel selected allowed each of Atto 488, CF®583R, and AZDyeTM 647 to be excited with separate, distinct lasers (488 nm, 560 nm, and 638 nm, respectively) and individual green (527/49 nm), orange (595/44 nm), and red (673/35 nm) emission filters, respectively. By contrast, CF®405S and CF®405L were both excited using the 405 nm laser and their emitted light separated using blue (445/30 nm) and green (527/49 nm) emission filters, respectively. These channel options represent only a subset of the combinations possible with Aplo Scope.

Imaging all five channels with their relevant laser-filter combinations at powers of ~1 mW and an exposure time of 100 ms yielded high-quality images of every target, with strong signal-to-noise in all cases and no cross-channel bleedthrough (Fig. 2). The two CF®405 dyes were clearly distinguishable despite their common excitation laser.

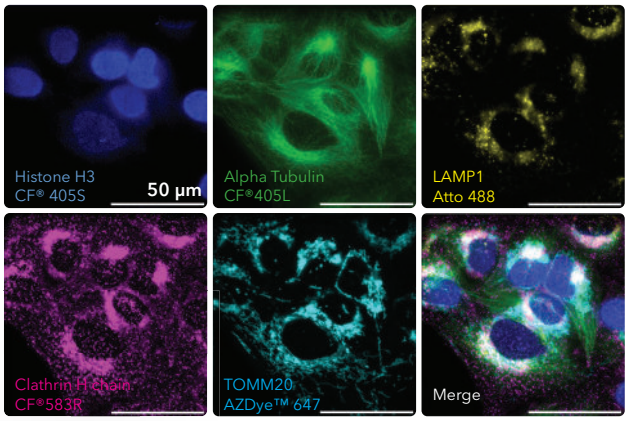


Figure 2 | Aplo Scope supports diverse multicolor imaging.

An example of U2OS cells probed for the five indicated targets with primary antibodies directly conjugated to their indicated fluorophores. Diffraction-limited widefield imaging was performed on Aplo Scope and images were processed and rendered in CODI.

Unlike other imaging platforms, Aplo Scope has been designed not only for easy and effective multicolor imaging but also high-performance super-resolution SMLM. Image acquisition by dSTORM, PALM, DNA-PAINT and other SMLM methods can be achieved using any suitable dyes due to the high-powered lasers, sensitive widefield acquisition, and real-time localization capabilities. Here, dSTORM of the TOMM20 and clathrin channels was performed in ONI's dSTORM Imaging Buffer to ensure efficient dye blinking and photostability, which allowed 20,000 frames of each dye to be acquired with minimal photobleaching. The large 110 x 110 µm field of view allowed localization to be performed over several cells simultaneously, and in total ~5-10 million localizations were recorded for each channel in each field. For both fluorophores, >85% of all detected localizations were localized to a precision of under 20 nm, and >55% to under 10 nm.

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Such data were of comfortably high quality to allow resolution of nanoscale biological structures. TOMM20 localization to the outer mitochondrial membrane could clearly be observed in cross-sections of whole mitochondria, while the structures of individual clathrin-coated pits were reported to a very high resolution (Fig. 3A). Due to the multifunctional capabilities of Aplo Scope, it was possible to acquire both SMLM and diffraction-limited imaging on the same sample, allowing the cellular and sub-cellular location of SMLM-acquired targets to be determined by reference to other molecules. Here, Histone H3, alpha-Tubulin, and LAMP1 were again imaged alongside the dSTORM channels. This enabled the dSTORM data to be put into its correct cellular context - for example, by comparing the mitochondrial TOMM20 signal to other organelles (nucleus and lysosomes); or by correlating clathrin pits to other cytoskeletal structures (microtubules) (Fig. 3B). Moreover, although here two SMLM channels were combined with three diffraction-limited channels, Aplo Scope supports SMLM of any suitable fluorophore such that three or more SMLM channels could be acquired instead.

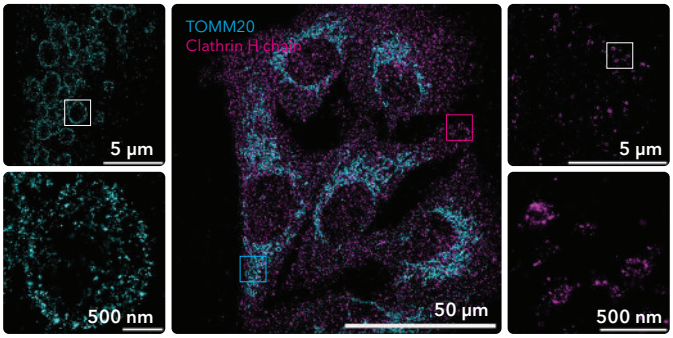
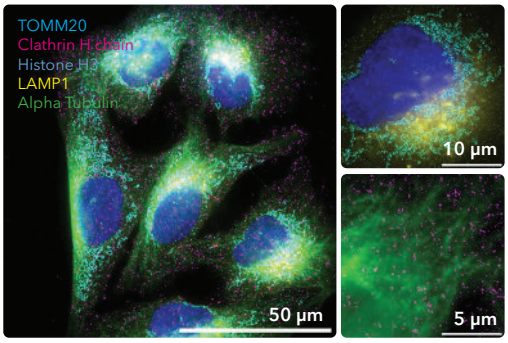


Figure 3 | Aplo Scope performs high-quality SMLM across a large field.

A) Example dSTORM data for anti-clathrin-CF®583R and anti-TOMM20-AZDyeTM 647 across several U2OS cells in a 110 x 110 µm field of view (center). Boxes indicate regions shown in zoomed single-channel examples (left, right).



B) Diffraction-limited images of anti-Histone H3-CF®405S, anti-Alpha Tubulin-CF®405L, and anti-LAMP1-Atto 488 overlaid with the two-color dSTORM data (left). The combination of SMLM and diffraction-limited data can provide cellular context for super-resolved targets, such by comparing organelle location (right, top) or cytoskeletal structures (right, bottom).

Solution with Aplo Scope

Aplo Scope, in combination with the cloud-based CODI, enable streamlined imaging of multiple cellular targets in a single experiment. Thanks to the combination of lasers and filters, the simultaneous imaging of different cellular targets is easily available. Here we demonstrate the detection of 5 different targets, however, in principle, detecting more targets is possible if appropriate affinity-based fluoropores are available.

For more information visit www.oni.bio

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