Efficient labeling of mitochondrial networks in micropatterned cells for toxicity studies

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- Optimized protocol for mitochondrial network labeling in both live and fixed cells
- Cell individualization and normalization thanks to adhesive micropatterns
- Straightforward comparison between different experimental conditions

Background

The architecture of the mitochondrial network is regarded as a good indicator of cell health, and is altered before the activation of the signaling pathways leading to cell death. Analysis of mitochondria alterations is therefore a crucial element of in vitro cell based toxicology studies. However, because of the high variability of cell morphology in classical 2D cell culture conditions, mitochondria network architecture remains challenging to analyze.

CYTOO’s 2D+ cell culture platform, based on adhesive micropatterns, offers control over the cells’ microenvironment, leading to normalized cell morphology and behavior.

By defining the 2D topology of cell adhesion, 2D+ Technology enables the fine control of the spreading and 3D shape of cultured cells, resulting in control of cell contractility, cell polarity, organelle positioning, or cell division axis. We demonstrate here the benefits of 2D+ in visualizing mitochondrial network architecture.

This Application Note describes CYTOO’s approach to visualize and analyze mitochondrial network architecture, on live and fixed cells, and alerts to possible pitfalls in mitochondrial staining protocols. Using this procedure, subtle changes in the mitochondrial network can be analyzed and the approach opens new perspectives in mitochondrial disorder exploration and toxicity studies.

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Method
Please note that this protocol is optimized for HeLa cells. Optimal micropattern protein coating as well as optimal concentrations of mitotracker may vary for other cell lines.

Normalize your cells on CYTOO micropatterns
Place a CYTOOchip 20x20 with fibronectin (FN) coated micropatterns into a CYTOO-chamber (optionally, labeled FN 650 micropatterns can be used to visualize the patterns). Seed 20,000 HeLa cells onto the mounted CYTOOchip in 500µl of culture media [MEM + glutamax (Invitrogen) with 10% FBS (Sigma), 0.5% penicillin/streptomycin (Gibco)]. After 2h of incubation (37°C, 5% CO2, humidified atmosphere), add 2.5ml of culture media to the 500µl in the CYTOOchamber and leave for 20h in the incubator.

Mitochondria staining
Mitochondria are stained in live and fixed cells by adding to the cell medium respectively Mitotracker Green FM (Invitrogen) at a final concentration of 20nM for 15min, or Mitotracker Deep Red FM (Invitrogen) at 100nM for 30min.

For live cell imaging
Wash out media containing the mitotracker, and image live cells by using a microscope under a controlled environment (humidified atmosphere, 5% CO2, 37°C), equipped with adequate fluorescence filters to visualize Mitotracker Green FM (Ex: 490nm, Em: 516nm) and eventually the FN650 micropatterns (Ex: 649nm, Em: 670nm). Note that Mitotracker Green is not retained by the mitochondria after cell fixation and can only be used on live cells.

For fixed cells
Wash out the media and fix cells in pre-warmed formalin solution 10% (Sigma). Stain Nuclei using 0.2 µg/ml Hoechst 33342 solution (Invitrogen) for 5min. Cells image using a microscope equipped with adequate fluorescence filters to visualize Mitotracker Deep Red FM (Ex: 644nm, Em: 665nm).

Characterization of mitochondria disruption due to cell fixation
Although live cell imaging remains the method of choice for the study of mitochondrial networks, it is difficult to apply this strategy in High Content Screening as it complicates overall workflow. Working with fixed cells is therefore often required. However, fixation is known to alter mitochondria network architecture. To illustrate this, quantification of mitochondrial network integrity from cells cultured 20 hours on FN micropatterns was carried out before and after formalin fixation. In live cell conditions, mitochondria networks stained with Mitotracker Deep Red (100nM, 30min) are completely preserved. In contrast, observation of the same cells after formalin fixation revealed a large proportion of cells with fragmented mitochondria (up to 45%, see Fig. 6). Similar results were obtained on micropatterns of various sizes and geometries as well as in standard culture conditions.

Potential experimental pitfalls
Avoid toxicity induced by the mitochondrial marker
As mitochondrial probes can be toxic to the cells when used above the optimal concentration, we recommend performing preliminary experiments by varying concentration and/or incubation times to define the range of toxicity of your mitochondrial marker.

As an example, we followed the integrity of mitochondria networks using epifluorescence microscopy after various treatments with Mitotracker Green FM. For this experiment, cells were seeded on CYTOO-chips Starter’s using the standard protocol (see method). After 24h, mitochondria were stained using increasing concentrations of Mitotracker Green FM (20nM to 200nM) for either 15min or 45min. High concentrations of Mitotracker Green FM (above 100nM) applied for a long period (45min) induce a high level of mitochondria network fragmentation showing that the mitochondrial marker is toxic for cells (Fig. 5A). However, by reducing Mitotracker concentration and time of exposure, such toxicity is avoided. Note that cell integrity is preserved on all micropattern sizes (see Fig. 5B).

Figure 5: (A) Potential toxicity of mitotracker treatment on micropatterned cells. Study based on a binary classification of mitochondria network integrity: Intact: no toxicity, Fragmented: toxicity. (B) Micropattern size has no effect on mitochondria network integrity. For both tests, representative pictures are shown at the bottom.
Application example: quantifying mitochondria dynamics in primary fibroblasts

In a recent paper, Chevrollier and coworkers \(^2\) used extra large Y micropatterns to precisely characterize mitochondria in steady-state live primary fibroblasts. After cell seeding on micropatterns, mitochondria were stained using Mitotracker Green FM and images were acquired under confocal microscopy. Based on the Filament Tracer tool from Imaris (Bitplane), they were able to measure the length of each mitochondria branch from the full network (See Fig. 7).

On one hand, micropatterns allowed the exquisite control of cell morphology, abrogating artifactual influence of cell shape on mitochondria extension. On the other, by custom-designing extra large adhesive Ys, the authors chose to maximize cell spreading and overall cell flatness, thus limiting mitochondria branch overlapping in the Z axis, and minimizing image segmentation errors.

Figure 7: Mitochondrial networks in primary fibroblasts (scale bars: 10 microns) The mitochondria were labeled with MitoTracker Green and deconvolved images were analyzed with Imaris Filament Tracer. A2: The color codes highlight the different lengths of the mitochondrial tubules between branch points. Details show the tips of mitochondrial tubules in green and the network branch points in red (Scale bars: 1 micron). Courtesy of Arnaud Chevrollier.

Conclusion

The present Application Note describes simple procedures to maximize the benefits of using CYTOO’s 2D+ technology when studying mitochondrial networks, both on live and fixed cells. Combined to mitochondrial markers, micropatterns offer a standardized way to analyze subtle changes in mitochondrial network architecture and dynamics upon different stimuli, by abrogating cell shape heterogeneity, and providing a straightforward comparison between different experimental conditions. This approach can be applied to a large panel of applications ranging from characterization of mitochondrial disorders to drug development and toxicity studies.

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Analysis of mitochondria can be carried out on all standard patterns (crossbow, disc, H, Y, L) as well as on custom-designed micropatterns. Please visit www.cytoo.com to see available products.