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## High content imaging of lysosomal phenotypes in iPSC-derived neurons using the Opera Phenix(TM) High-content Screening System

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### ABSTRACT

This protocol describes the use of the Opera Phenix high-content screening system and its advanced software Harmony 5.1 to image and analyse live and fixed neuronal cell cultures for the measurement and characterization of lysosomal phenotypes. This includes quantifying lysosomal size and number using a lysosome staining reagent, quantifying lysosomal activity using DQ red-BSA uptake and processing in live cells, and quantifying autophagic flux using P62 and LC3 immunofluorescent staining in the presence and absence of the autophagy-lysosomal pathway inhibitor Bafilomycin A1. The aim of this protocol is to create an automated analysis pipeline that can be used to process large volumes of data for any cell culture for the measurement of lysosomal and autophagosomal phenotypes under different treatment conditions. The use of 3 different and complementary approaches allows of a flexible and tailorabile protocol with the advantage that any of these 3 analyses can be used separate or in parallel.

### MATERIALS

#### **Key equipment/consumables/reagents/solutions**

Cultures are grown, treated, and stained following the previous protocol "Culture and staining of iPSC-derived neurons for lysosomal phenotype analysis" (ref of the protocol).

#### **Material input (animal, cell, tissue, fraction details)**

This protocol can be applied to different cell types for the assessment of lysosomal functions. Here we apply it to induced pluripotent stem cells differentiated into Ventral Medial Dopaminergic neurons (protocol available at [10.17504/protocols.io.bu7ynzpw](https://dx.doi.org/10.17504/protocols.io.bu7ynzpw)) or cortical neurons (protocol available at [10.17504/protocols.io.bu6znzf6](https://dx.doi.org/10.17504/protocols.io.bu6znzf6)). Cortical neurons were cultured until DIV50 and ventral medial dopaminergic neurons cultured until DIV40.

**Protocol status:** Working  
We use this protocol and it's working

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## BEFORE START INSTRUCTIONS

### Experimental Outline

As stated in the staining protocol, the imaging can be adjusted depending on the conditions and specification of the culture to be analysed. Specifically, we use 2 different imaging paradigms in this protocol:

For live imaging analysis cells are imaged 15, 45 and 90 minutes after adding the probes using the Opera Phenix high-content screening system. Hoechst, Alexa488 and Alexa561 Laser/filter pairs are used for DQ-red BSA treatment imaging. Hoechst, Alexa488, Alexa561 and Alexa647 laser/filter pairs are used to image Mitotracker/Lysosomal probes. Images are taken with 40x water objective, 3 z-steps, at least 25 fields of view, and the plate is kept under cell culture conditions (37°C, 5% CO<sub>2</sub>).

Note: 40x objective is needed to obtain enough detail for accurate Lysosome-Mitophagy analysis. Z-step and fields of view are selected to obtain enough images without compromising the time it takes to finish a round of imaging.

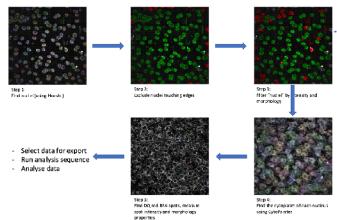
For fixed cultures analysis cultures are imaged with 40x water objective, 10 z-steps (0.5mm step size as recommended by the manufacturer), at least 46 fields of view per well (covering 16% of the well's area).

Note: Imaging conditions are selected taking into consideration the detail needed (analysis of organelles need higher magnification), and the minimum number of cells needed to obtain a robust result (if the culture has very little number of cells, more fields of view could be needed). Please refer to the Harmony software manual (<https://www.perkinelmer.com/uk/product/harmony-4-9-office-license-hh17000010>) for assistance in setting imaging parameters.

## Methods section 1

### 1 Example analysis sequence:

#### 1. Lysosomal degradation capacity using DQ-red-BSA:



#### Step 1:

Using the “find nuclei” analysis block, with the channel in which the nuclei are stained (we used Hoechst) the user can choose the most accurate method out of 4 available and adjust parameters like threshold and

splitting sensitivity for optimal Nuclei detection. The user can choose the best method based on visual feedback from an image containing nuclei (refer to Harmony software manual section 3.4)

**Step 2:**

Nuclei touching the side of the image can be excluded using “select population” à “common filter” à “exclude on edge”. (Refer to Harmony software manual section 3.21)

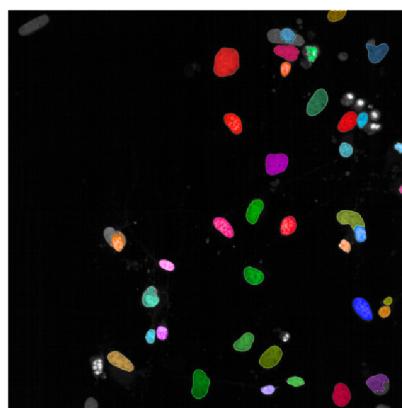
**Step 3:**

Nuclei are filtered by size, roundness, and intensity: first intensity and morphology properties of the nuclei can be measured using “calculate intensity properties” and “calculate morphology properties” in the nuclei population and used to exclude unwanted objects through “select population” and “filter by property”. (Refer to Harmony software manual section 3.21)

Size exclusion: very small nuclei usually belong to dead cells, they can be filtered out by picking a lower limit size threshold, and in some cases nuclei that are attached together are not successfully recognised as individual nuclei and separated by splitting, so upper limit size exclusion allows us to filter those out.

Roundness exclusion: some non-specific DAPI signals can be filtered out by roundness exclusion, as well as multiple nuclei that were not split properly (roundness of a particle ranges between 0 and 1, user can pick a cut-off value using the software’s visual feedback)

Intensity exclusion: very intense DAPI stain usually occurs in nuclei of dead cells, filtering out dead cells using two filters (size and intensity) allows for a better, more selective exclusion.

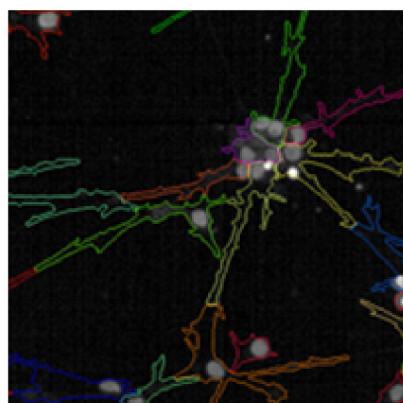
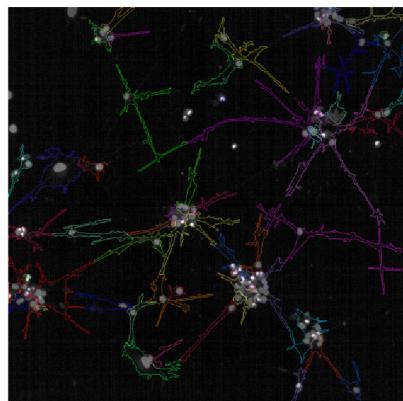


Example of nuclei selected and filtered

**Step 4:**

Using the “find cytoplasm” analysis block, with the channel corresponding to the cytoplasmic stain (we used Cytopainter green), the user can choose one of several available methods to detect the cytoplasm region of the cells. As in the case of the “find nuclei” block, this process can be fine-tuned by changing parameters like

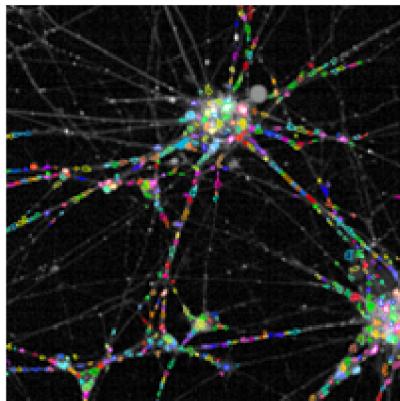
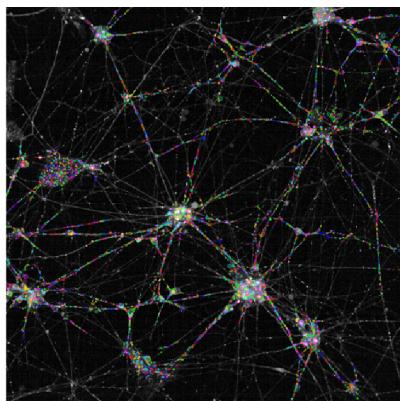
threshold, splitting sensitivity, etc. the user can choose the best method based on visual feedback from an image containing cells. (Refer to Harmony software manual section 3.5)



Example of cytoplasm area selected (note how only filtered nuclei are considered to have a cytoplasm)

### Step 5:

Using the “find spots” analysis block and using the correct channel in which the spots are stained (DQ-red BSA), the user can detect spots using one of the available methods. Finding the spots can be restricted to the area of previously detected cytoplasm by picking the cytoplasm region. Like previous steps, this process can be altered by fine-tuning parameters like threshold for detecting spots based on their overall brightness, or brightness relative to background, while also restricting the size of the spots to avoid including large non-specific signals, the best method can then be chosen based on visual feedback from images containing spots (Refer to Harmony software manual section 3.6).



Example of spots detected (note how spots are only detected in previously detected as cytoplasmic areas)

Intensity and morphology properties of the spots can now be measured using “calculate intensity properties” and “calculate morphology properties” for the “spots” population, the user can select the variables they wish to measure out of a large list. Example: for intensity measures, user can select variables like mean, Sum, Min, Max, Std Dev, etc. for morphology measures variables include spot size, perimeter, roundness, diameter, etc.

#### Step 6:

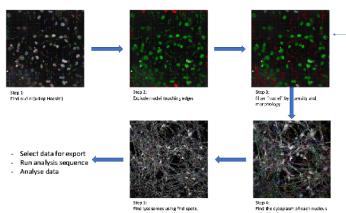
In the “define result” section, the user must select all the variables that they wish to obtain at the end of the analysis, a list of all the measured variables will be available in this menu and the user can manually select the variables they want.

Then all the Z-planes, fields of view and wells where the user wants to run the analysis must be selected before clicking on the “Evaluation” tab and starting the measurement.

Output data is displayed in a table that can be saved or exported to Excel for analysis.  
(Refer to Harmony software manual section 3.29).

Examples of data generated using this analysis sequence is provided below (example 1)

## 2. Measuring lysosome number and size using Lysosomal staining reagent (Cytopainter orange)

**Step 1:**

Using the “find nuclei” analysis block, with the channel in which the nuclei are stained (we used Hoechst) the user can choose the most accurate method out of 4 available and adjust parameters like threshold and splitting sensitivity for optimal Nuclei detection. The user can choose the best method based on visual feedback from an image containing nuclei (refer to Harmony software manual section 3.4)

**Step 2:**

Nuclei touching the side of the image can be excluded using “select population” à “common filter” à “exclude on edge”. (Refer to Harmony software manual section 3.21)

**Step 3:**

Nuclei are filtered by size, roundness, and intensity: first intensity and morphology properties of the nuclei can be measured using “calculate intensity properties” and “calculate morphology properties” in the nuclei population and used to exclude unwanted objects through “select population” and “filter by property”. (Refer to Harmony software manual section 3.21)

Size exclusion: very small nuclei usually belong to dead cells, they can be filtered out by picking a lower limit size threshold, and in some cases nuclei that are attached together are not successfully recognised as individual nuclei and separated by splitting, so upper limit size exclusion allows us to filter those out.

Roundness exclusion: some non-specific DAPI signals can be filtered out by roundness exclusion, as well as multiple nuclei that were not split properly (roundness of a particle ranges between 0 and 1, user can pick a cut-off value using the software’s visual feedback)

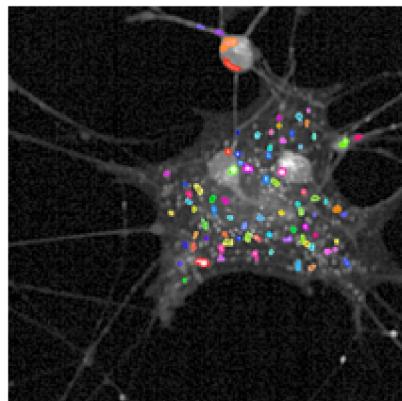
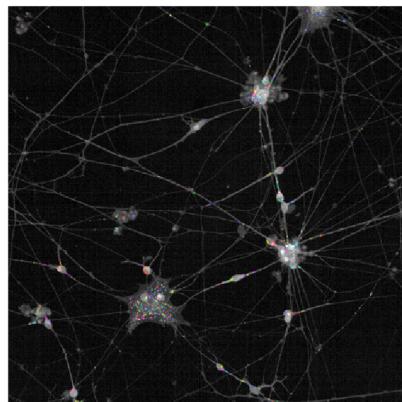
Intensity exclusion: very intense DAPI stain usually occurs in nuclei of dead cells, filtering out dead cells using two filters (size and intensity) allows for a better, more selective exclusion.

**Step 4:**

Using the “find cytoplasm” analysis block, with the channel corresponding to the cytoplasmic stain (we used Cytopainter green), the user can choose one of several available methods to detect the cytoplasm region of the cells. As in the case of the “find nuclei” block, this process can be fine-tuned by changing parameters like threshold, splitting sensitivity, etc. the user can choose the best method based on visual feedback from an image containing cells. (Refer to Harmony software manual section 3.5)

**Step 5:**

Using the “find spots” analysis block and using the correct channel in which the spots are stained (Lysosomal staining Cytopainter orange), the user can detect spots using one of the available methods. Finding the spots can be restricted to the area of previously detected cytoplasm by picking the cytoplasm region. Like previous steps, this process can be altered by fine-tuning parameters like threshold for detecting spots based on their overall brightness, or brightness relative to background, while also restricting the size of the spots to avoid including large non-specific signals, the best method can then be chosen based on visual feedback from images containing spots (Refer to Harmony software manual section 3.6).



Example of Lysosomes detected inside the cytoplasm

Intensity and morphology properties of the spots can now be measured using “calculate intensity properties” and “calculate morphology properties” for the “spots” population, the user can select the variables they wish to measure out of a large list. Example: for intensity measures, user can select variables like mean, Sum, Min, Max, Std Dev, etc. for morphology measures variables include spot size, perimeter, roundness, diameter, etc.

#### Step 6:

In the “define result” section, the user must select all the variables that they wish to obtain at the end of the analysis, a list of all the measured variables will be available in this menu and the user can manually select

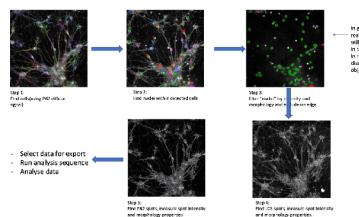
the variables they want.

Then all the Z-planes, fields of view and wells where the user wants to run the analysis must be selected before clicking on the “Evaluation” tab and starting the measurement.

Output data is displayed in a table that can be saved or exported to Excel for analysis.  
(Refer to Harmony software manual section 3.29).

Examples of data generated using this analysis sequence is provided below (example 2)

### 3 Measuring autophagy flux by analysing P62 and LC3 staining in fixed cells:



The steps of the following analysis sequence are little bit different to the previous two, due to the absence of the Cytopainter green cytoplasmic stain to detect cytoplasms

#### Step 1:

Using the “find cells” analysis block with the channel stained with P62, cells can be detected using the diffuse signal of P62 present in the cytoplasm (this is different from the P62 puncta or spots quantified in step 5). The user can choose the most accurate method out of 5 available and adjust parameters like threshold and splitting sensitivity for optimal cell detection. The user can choose the best method based on visual feedback from an image containing nuclei (refer to Harmony software manual section 3.7). this will create a “cell” population that can be selected as a region of interest (ROI) in further steps.

#### Step 2:

Using the “find nuclei” analysis block, with the channel in which the nuclei are stained (we used DAPI) the user can choose the most accurate method out of 4 available and adjust parameters like threshold and splitting sensitivity for optimal Nuclei detection. The user can choose the best method based on visual feedback from an image containing nuclei (refer to Harmony software manual section 3.4). nuclei detection is restricted to select ROI.

#### Step 3:

Nuclei touching the side of the image can be excluded using “select population” à “common filter” à “exclude on edge”. (Refer to Harmony software manual section 3.21)

Nuclei are filtered by size, roundness, and intensity: first intensity and morphology properties of the nuclei can be measured using “calculate intensity properties” and “calculate morphology properties” in the nuclei population and used to exclude unwanted objects through “select population” and “filter by property”. (Refer to Harmony software manual section 3.21)

**Step 4:**

Using the “find spots” analysis block and using the correct channel in which the spots are stained (LC3), the user can detect spots using one of the available methods. Finding the spots can be restricted to the area of previously detected cells by picking the cytoplasm ROI. Like previous steps, this process can be altered by fine-tuning parameters like threshold for detecting spots based on their overall brightness, or brightness relative to background, while also restricting the size of the spots to avoid including large non-specific signals, the best method can then be chosen based on visual feedback from images containing spots (Refer to Harmony software manual section 3.6).

Intensity and morphology properties of the spots can now be measured using “calculate intensity properties” and “calculate morphology properties” for the “spots” population, the user can select the variables they wish to measure out of a large list. Example: for intensity measures, user can select variables like mean, Sum, Min, Max, Std Dev, etc. for morphology measures variables include spot size, perimeter, roundness, diameter, etc.

**Step 5:**

Using the “find spots” analysis block and using the correct channel in which the spots are stained (P62) the user can detect spots using one of the available methods. Finding the spots can be restricted to the area of previously detected cytoplasm by picking the cytoplasm region. Like previous steps, this process can be altered by fine-tuning parameters like threshold for detecting spots based on their overall brightness, or brightness relative to background, while also restricting the size of the spots to avoid including large non-specific signals, the best method can then be chosen based on visual feedback from images containing spots (Refer to Harmony software manual section 3.6).

Intensity and morphology properties of the spots can now be measured using “calculate intensity properties” and “calculate morphology properties” for the “spots” population, the user can select the variables they wish to measure out of a large list. Example: for intensity measures, user can select variables like mean, Sum, Min, Max, Std Dev, etc. for morphology measures variables include spot size, perimeter, roundness, diameter, etc.

## Step 6:

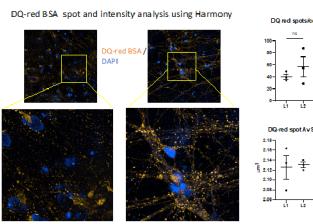
In the “define result” section, the user must select all the variables that they wish to obtain at the end of the analysis, a list of all the measured variables will be available in this menu and the user can manually select the variables they want.

Then all the Z-planes, fields of view and wells where the user wants to run the analysis must be selected before clicking on the “Evaluation” tab and starting the measurement.

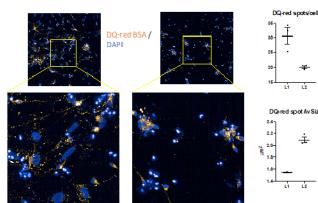
Output data is displayed in a table that can be saved or exported to Excel for analysis.  
(Refer to Harmony software manual section 3.29).

Examples of data generated using this analysis sequence is provided below (example 3)

## Example 1:

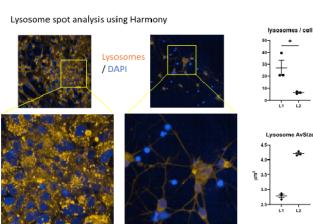


Example images showing DQ-red BSA fluorescence in two different cortical differentiated cell lines. L1 is an example of low DQ-red fluorescence and is indicative of reduced lysosomal activity. L2 is an example of higher DQ-red fluorescence and is indicative of increased lysosomal activity. The graphs show the analysis of these lines for three technical replicates.

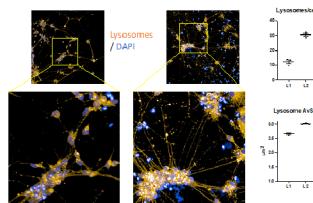


Example images showing DQ-red BSA fluorescence in two different ventral medial differentiated cell lines. L1 is an example of low DQ-red fluorescence and is indicative of reduced lysosomal activity. L2 is an example of higher DQ-red fluorescence and is indicative of increased lysosomal activity. The graphs show the analysis of these lines for three technical replicates.

## Example 2:

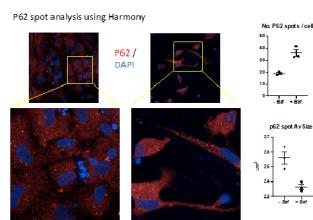


Example images showing Lysosomal staining cytopainter orange fluorescence in two different cortical differentiated cell lines. L1 is an example of high Lysosomal staining cytopainter orange fluorescence and shows more lysosomes per cell. L2 is an example of lower Lysosomal staining cytopainter orange fluorescence and is indicative of less lysosomes per cell. The graphs show the analysis of lysosomal number and size for these lines for three technical replicates.

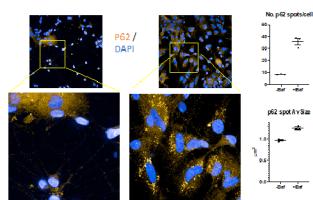


Example images showing Lysosomal staining cytopainter orange fluorescence in two different ventral medial differentiated cell lines. L1 is an example of lower Lysosomal staining cytopainter orange fluorescence and is indicative of less lysosomes per cell. L2 is an example of high Lysosomal staining cytopainter orange fluorescence and shows more lysosomes per cell. The graphs show the analysis of lysosomal number and size for these lines for three technical replicates.

### Example 3:



Example images showing P62 fluorescence in one cortical differentiated cell line treated with or without bafilomycin A1. Bafilomycin treatment has increased the number of P62 puncta per cell and this is shown in the graphs for three technical replicates.



Example images showing P62 fluorescence in one ventral medial differentiated cell line treated with or without bafilomycin A1. Bafilomycin treatment has increased the number and size of P62 puncta per cell and this is shown in the graphs for three technical replicates.

Gantner, C. W., et al. (2020). "An optimized protocol for the generation of midbrain dopamine neurons under defined conditions." *Star Protocols* 1(2): 100065.

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