METHODOLOGY FOR QUANTIFY CELL VIABILITY AFTER ACUTE EXPOSURE OF AMYLOID BETA OLIGOMERS (AβO) ON HUMAN NEURONS
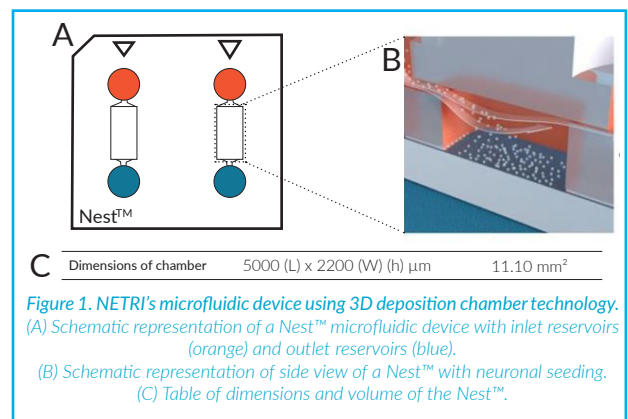
APPLICABLE CHIPS REFERENCES

- Nest™
- DuaLink™
- DuaLink™ Ultra

- DuaLink™ Delta Ultra
- DuaLink™ Shift
- TriaLink™

- TriaLink™ Ultra
- Duplex™

PURPOSE

This application protocol describes a methodology to quantify the effect of β-amyloid oligomers (AβO), from ETAP-Lab®, exposed on neurons seeded in NETRI's microfluidic devices. The aim is to examine neurotoxicity on human cells of AβO, involved in the Alzheimer's disease.

This protocol can be used with animal primary cells (Application Note - Evaluation of amyloid beta oligomers (AβO) effects on functional network integrity of rodent hippocampal neurons), cell lines and neurons derived from human induced pluripotent stem cells (hiPSCs).

Here, glutamatergic neurons iPSC-derived are seeding in a 3D-deposition chamber technology, called Nest™ device, using our previously published protocol (Operating Protocol - UniFluidics™ – Nest). 3D-deposition chamber technology (Nest™), is useful to avoid sheer stress of media change and flow fluid and thus, biases in peptide injury effect (Figure 1) (Maisonneuve et al. 2021). The level of neurotoxicity has been measured by quantifying the ratio of alive vs death cells after acute exposure. Cell viability has been quantified using LIVE/DEAD™ Viability/Cytotoxicity (LDA, Thermo Fisher Scientific Inc, L3211) in NETRI's microfluidic devices using our protocol described elsewhere (c.f. Operating_Protocol - ImmunoStaining).

Figure 1. NETRI's microfluidic device using 3D deposition chamber technology.
(A) Schematic representation of a Nest™ microfluidic device with inlet reservoirs (orange) and outlet reservoirs (blue).
(B) Schematic representation of side view of a Nest™ with neuronal seeding.
(C) Table of dimensions and volume of the Nest™.

Dimensions of chamber — 5000 (L) x 2200 (W) (h) µm — 11.10 mm²

MATERIALS

- NETRI's microfluidic device Nest™
- Glutamatergic neurons iPSC-derived from FCDI (iCell GlutaNeurons, 01279, Catalog #: R1061)
- Amyloid beta oligomers (AβO) from ETAP- Lab at a concentration of 500 µM (Mota,

- LIVE/DEAD™ Viability/Cytotoxicity kit (Thermo Fisher Scientific Inc, L3211)
- Software Fiji with ImageJ core 1.53e (Schindelin et al. 2012)
- Graph Pad Prism 8
- Computer with Windows/Linux/MacOs compatible with Fiji

PROCEDURE

All the below steps are performed according to the Good Laboratory Practices and under laminar flow hood.

1. AβO preparation

1.1. Thawing aliquot of AβO solution at room temperature.
1.2 Rapidly centrifugate aliquot of AβO solution.

2. AβO exposure on human neurons

2.1. Following the required concentration according to media change to dilute the solution in the neuronal culture media (Table 1).
2.2. Performing classical media change with peptide solutions instead of media cf. operating protocol in 3D Deposition chamber.

Note: In this study, the peptide exposure has been performed at day 13 on human glutamatergic neurons. For other cell types and/ or specific scientific questions cf. your expertise or contact us for scientific advice.

Required concentration (µM)	Concentration prepared for a 50% media change (µM)	Concentration prepared for a 75% media change (µM)
1	2	1.5
5	10	7.5
10	20	15
100	200	150

Table 1. Corresponding concentration of AβO according to media change percentage.

Note: Please refer to media change at targeted days. Example: At D13, glutamatergic neurons have a 50% media change.

3. LDA assays and fluorescent pictures

3.1. Perform LDA assays 24h after AβO peptide application on neurons.
3.2. Acquire pictures with epifluorescent microscopy of the whole 3D-deposition chamber.
3.3. Save pictures in "tiff" format.

Note: Please refer to Operating_Protocol - ImmunoStaining.

4. Select ROI of images of interest

4.1. Open Fiji, keep it as a small window
4.2. Drag and drop the image of alive cells in the "Fiji" window
4.3. Select "Rectangle selection" to make an ROI
4.4. Press "T" touch
4.5. Click on "Image > Duplicate..." or Press "Shift + Ctrl + D" to duplicate the ROI picture of live cells
4.6. Drag and drop the image of dead cells in the "Fiji" window
4.7. Apply ROI previously saved on the image of dead cells
4.8. Click on "Image > Duplicate..." or Press "Shift + Ctrl + D" to duplicate the ROI picture of dead cells

Note: It is important to have the exact same ROI on both pictures (live and dead cells).

5. Merge of live and dead cells pictures

5.1. Click on "Image > Adjust > Brightness/Contrast"
5.2. Adjust the parameters to obtain the best visualization, start with Auto (Figure 2A)
5.3. On live cells pictures: click on "Image > Lookup Tables" and select "Green"
5.4. On dead cells pictures: click on "Image > Lookup Tables" and select "Red"
5.5. Click on "Image > Color > Merge channels..." (Figure 2B)
5.6. Select dead cell picture for channel "C1 (red)" (Figure 2B)
5.7. Select live cell picture for channel "C2 (green)" (Figure 2B)
5.8. Select "Keep source images" if you want to keep both pictures independently. (Figure 2B)
5.9. Click on "OK". (Figure 2B)

Note: By consensus, live cells pictures are represented in green fluorescence and dead cells picture, in red fluorescence.
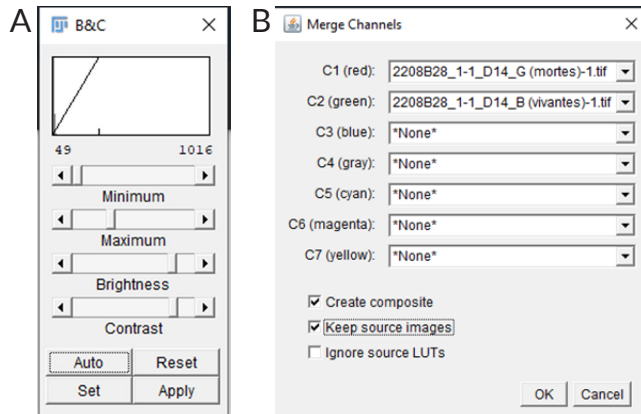

Figure 2. (A) *Adjusting Brightness/Contrast to enhance visualization of neurons.* **(B)** *Selecting images for each color channels for merging pictures.*

6. Save the pictures

6.1. On the "Fiji" window, click on "File"
6.2. Select "Save as".
6.3. Choose the picture format: Tiff/PNG/Jpeg
6.4. Select folder and name of the merged picture.

Note: " Tiff" format is used for imaging processing while PNG and Jpeg are used for illustrative pictures.

7. Binarization of duplicated pictures for cell quantification for live cells picture

7.1. Click on the picture of live cells
7.2. Click on "Image > Adjust > Threshold" or Press "Shift + Ctrl + T"
7.3. Adjust the binarization with the "Threshold" window (Figure 3): Keep "Default and Red".
7.4. Click on "Auto" and adjust, if necessary, to avoid background (Figure 4B)
7.5. Click on "Apply" (Figure 4C)
7.6. Choose "Process > Binary > Watershed" (Figure 5)
7.7. Click on "Analyze > Analyze Particles"
7.8. Enter parameters in Figure 6B
7.9. Click on "OK": the "Summary" window appears corresponding to results (Figure 7)

8. Binarization of duplicated pictures for cell quantification for dead cells picture

8.1. Click on the picture of dead cells
8.2. Click on "Image > Adjust > Threshold" or Press "Shift + Ctrl + T"
8.3. Adjust the binarization with the "Threshold" window (Figure 3): Keep "Default and Red".
8.4. Click on "Auto" and adjust, if necessary, to avoid background (Figure 4B)
8.5. Click on "Apply" (Figure 4C)
8.6. Choose "Process > Binary > Watershed" (Figure 5A)
8.7. Click on "Analyze > Analyze Particles"
8.8. Enter parameters in Figure 6B
8.9. Click on "OK": the "Summary" window appears corresponding to results (Figure 7)
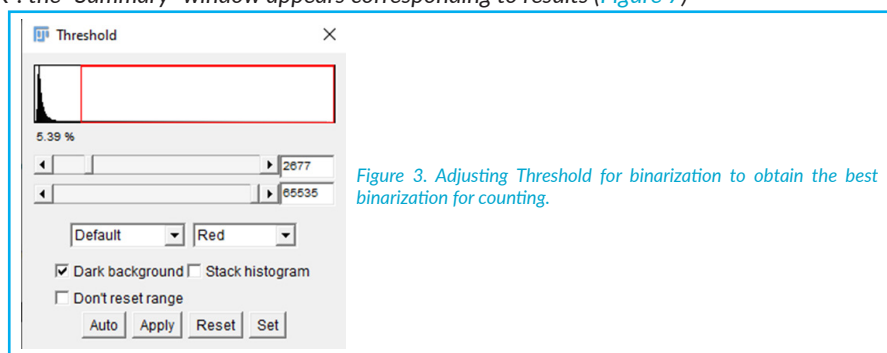

Figure 3. Adjusting Threshold for binarization to obtain the best binarization for counting.
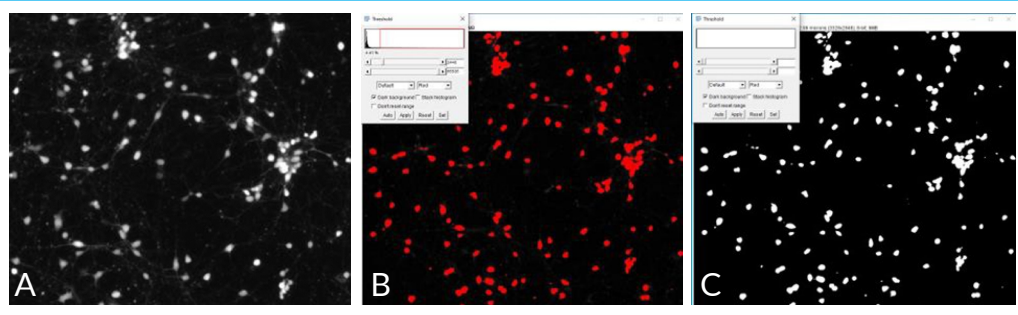
Figure 4. Pictures of live cells (A) before binarization, (B) after clicked on "Auto" and (C) after clicked on "Apply".
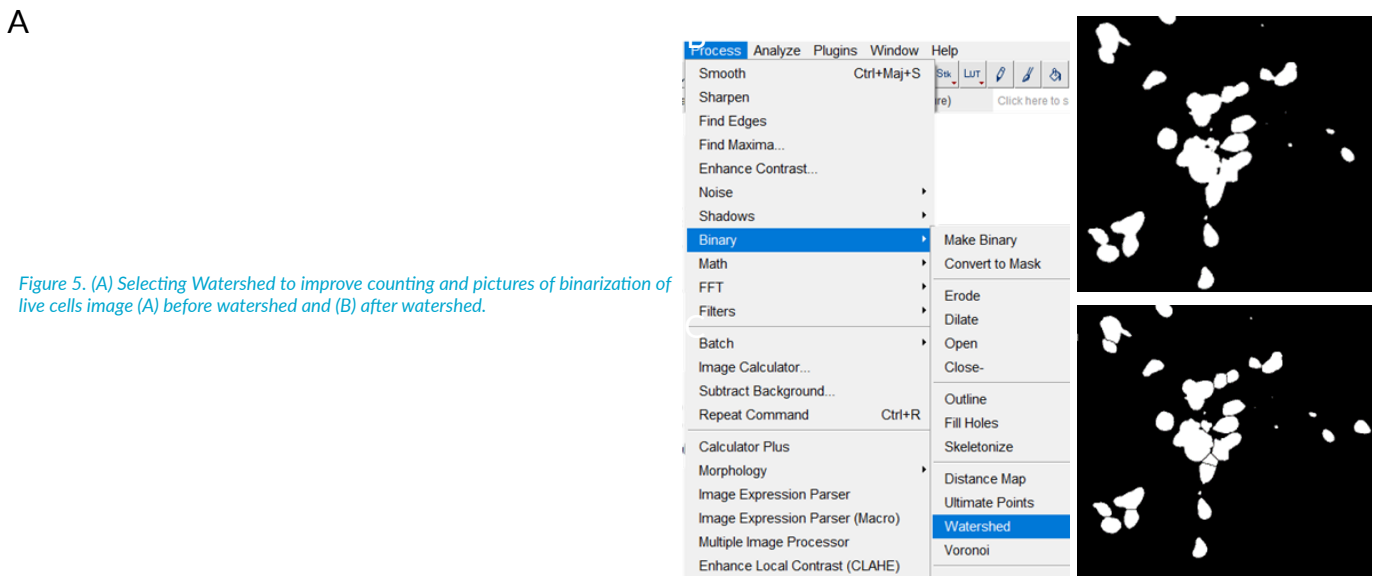

Figure 5. (A) Selecting Watershed to improve counting and pictures of binarization of live cells image (A) before watershed and (B) after watershed.
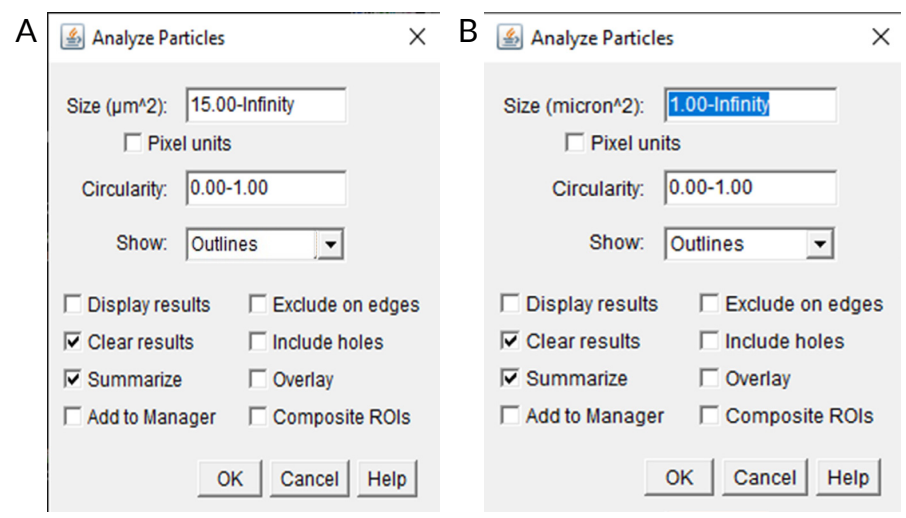

Figure 6. Parameters to count particles binarized on pictures of interest. (A) Parameters for Alive cells analysis. (B) Parameters for Dead cells analysis.

9. Copy raw data to save the measurement.

9.1. On the "Results" windows, click on "File"

9.2. Select "Save As".

10. Save the pictures.

10.1. On the "Fiji" window, click on "File"

10.2. Select "Save as".

11. Statistical analyses of LDA results.

11.1. Using a statistics and graphs software as GraphPad Prism or Microsoft Excel.

12. S12. Computation of Live/Dead ratio.

12.1. To have total cell count on both pictures: $\text{Total number of cells} = \dfrac{\text{count of live cells}}{\text{count of dead cells}}$

12.2. To have a ratio of live cells: $\% \text{ of } Live \text{ cells} = \dfrac{\text{count of live cells}}{\text{total number of cells}}$

12.3. To have a ratio of dead cells: $\% \text{ of } Dead \text{ cells} = \dfrac{\text{count of dead cells}}{\text{total number of cells}}$

Expected Results

Figure 7. Quantitative results of cells counting on live cells and dead cells pictures.
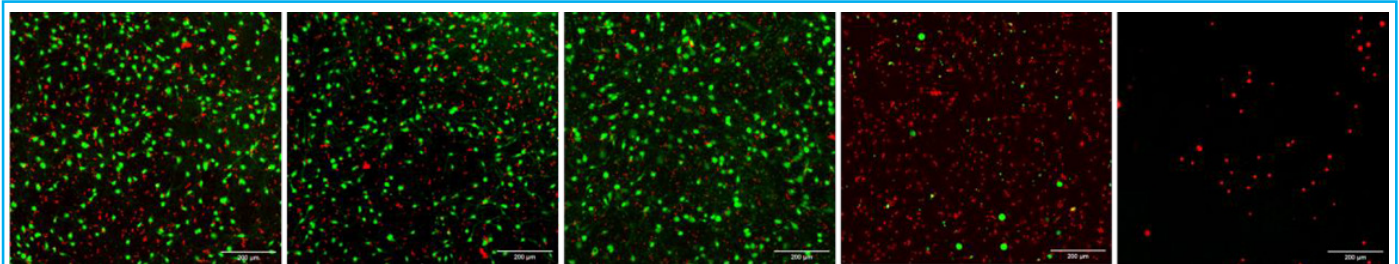

Figure 8. Fluorescent pictures of LDA assays according to different concentration of AβO exposure from 0 μM to 100 μM. Green fluorescent correspond to live cells and red fluorescence correspond to dead cells.

TROUBLESHOOTING

Troubles	Solutions
Doubt about the counting	• Have the same ROI on both pictures • Modify binarization on both pictures to refine the analysis after optimization of brightness and contrast • Make sure that pictures are not blurred
Cell homogeneity is not ideal	• Perform LDA analysis with several and different ROI on both pictures.

Table 2. List of potential issues that can happen and proposed solutions and advice.

1. Maisonneuve, B G C, L Libralesso, L Miny, A Batut, J Rontard, M Gleyzes, B Boudra, and Thibaut Honegger. 2021. "Deposition Chamber Technology as Building Blocks for a Standardized Brain-on- Chip Framework." BioRxiv.
2. Mota, Sandra I., Ildete L. Ferreira, and A. Cristina Rego. 2014. "Dysfunctional Synapse in Alzheimer's Disease - A Focus on NMDA Receptors." Neuropharmacology 76 (PART A): 16–26. https://doi.org/10.1016/j.neuropharm.2013.08.013.
3. Schindelin, Johannes, Ignacio Arganda-Carreras, Erwin Frise, Verena Kaynig, Mark Longair, Tobias Pietzsch, Stephan Preibisch, et al. 2012. "Fiji: An Open-Source Platform for Biological-Image Analysis." Nature Methods. Nat Methods. https://doi.org/10.1038/nmeth.2019.

CONTACT

www.netri.com

NETRI SAS - 321 Avenue Jean Jaures 69007 Lyon - FRANCE
Bat. A - 213 Rue de Gerland 69007 Lyon - FRANCE
Phone: +33 4 78 23 08 66 - Email: contact@netri.com