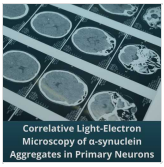

Apr 07, 2021

Correlative Light-Electron Microscopy of α-synuclein Aggregates in Primary Neurons

Felix Kraus[1], vtrinkaus [1], Ruben Fernandez Busnadiego[1]

[1]Harvard Medical School, Department of Cell Biology

1 *Works for me* dx.doi.org/10.17504/protocols.io.btyinpue

Felix Kraus

ABSTRACT

This protocol details methods for correlative light-electron microscopy of α-synuclein aggregates in primary neurons.

DOI

dx.doi.org/10.17504/protocols.io.btyinpue

PROTOCOL CITATION

Felix Kraus, vtrinkaus , Ruben Fernandez Busnadiego 2021. Correlative Light-Electron Microscopy of α-synuclein Aggregates in Primary Neurons. **protocols.io** https://dx.doi.org/10.17504/protocols.io.btyinpue

KEYWORDS

α-synuclein, aggregates, microscopy, light-electron, light, electron, SEM, CorrSight, FIB-SEM

CREATED

Apr 05, 2021

LAST MODIFIED

Apr 07, 2021

OWNERSHIP HISTORY

Apr 05, 2021 Liz Brydon Protocols.io

Apr 07, 2021 Felix Kraus

PROTOCOL INTEGER ID

48874

GUIDELINES

This protocol is written for the following instruments:
PDC-3XG Plasma Cleaner, Harrick
Vitrobot, Thermofisher
CorrSight, Thermofisher with software MAPS Version 2.1
Quanta or Scios Dual-Beam FIB-SEM, Thermofisher

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

BEFORE STARTING

Prerequisites: Protein of interest (e.g. α-Syn) or surrogate marker should be tagged with fluorescent protein (e.g. GFP).

Correlative Light-Electron Microscopy of α-synuclein Aggregates in Primary Neurons 40s

1 Grow primary neurons on EM grids (SiO2, R1/4 or 1.2/20, Quantifoil):

Carbon Au grids may also be used, but they should be coated with an additional layer of carbon to stabilize the grids and to allow the neurons attach better on the grids.

1.1 Before using the EM grids, glow discharge them for **00:00:30** at highest voltage (PDC-3XG Harrick plasma cleaner) and subsequently coat with [M]**1 mg/ml poly-D-lysine** and [M]**1 microgram per milliliter (µg/mL) laminin** . 30s

1.2 To have a sufficient number of neurons on the grids, plate 120,000 neurons per well of a 24-well plate.

2 Transduce neurons with vector / do seeding experiment.

3 Plunge freeze the neurons:

3.1 First, exchange the medium to neuronal medium containing 10 % glycerol to ensure good vitrification (see also Baeuerlein et al., Cell, 2017).

3.2 Hold the grids carefully with plunging tweezers and insert the tweezers in the holder of the Vitrobot (Thermofisher). Make sure grids are blotted from the front as well as from the back with Whatman paper. Use a humidity of 80 % and a temperature of **37 °C** , a blot force of 10 and blot time of **00:00:10** . 10s

Prepare the Vitrobot prior to exchanging the neuronal medium to ensure fast plunge-freezing and only short incubation of the neurons in 10 % glycerol (around 5 min). For preparing the Vitrobot, turn it on, apply the settings you want to use, add liquid nitrogen (LN_2) into the LN_2container and liquefy the ethane / propane gas in the cavity that is cooled by LN_2).

4 After plunging, store EM grids in appropriate EM grid boxes in LN_2.

Citation: Felix Kraus, vtrinkaus , Ruben Fernandez Busnadiego (04/07/2021). Correlative Light-Electron Microscopy of ÃÂ±-synuclein Aggregates in Primary Neurons. https://dx.doi.org/10.17504/protocols.io.btyinpue

5 For cryo-fluorescence microscopy, clip EM grids in autogrid holders with cut-out to enable FIB-milling under LN_2. Place clip ring on top of EM grid in the back of the autogrid frame, cells facing downwards to enable FIB-milling of the cells later on.

6 Transfer clipped grids into shuttles of the CorrSight (Thermofisher) microscope. Cells facing again downward.

7 Transfer shuttles into CorrSight shuttle boxes and bring the grids to the microscope.

8 Move the shuttle into the cryo-box of the cooled-down CorrSight microscope.

For cooling down, the tank in the back of the cryo-box is filled with LN_2 and the slowly evaporating LN_2 gas is led through the box to the outside, cooling the atmosphere in the box to a temperature of -180 °C.

9

Open the LA software and the MAPS software, create a new project in MAPS and find areas of interest in the specimen at 5 x magnification. Take overview images of the EM grids in DIC mode as well as in fluorescence mode (choose channel depending on your fluorophore).

10

Find cells that look promising (they should be close to the center of the grid and should be located in the middle of a square but not on the EM grid bar) and acquire 20 x or 40 x images, depending on the size of the feature of interest. The MAPS project will be saved automatically.

11 After imaging of the grids, take them carefully out of the cryo-box and place them in LN_2.

12 Move the MAPS project to the computer of the FIB instrument (Scios or Quanta, Thermofisher).

13 Transfer the EM grids from the CorrSight shuttles into the FIB shuttle under LN_2. Grids should be inserted into the shuttle with cells facing towards the experimenter and the cut-out should face upright. Transfer the EM grids subsequently under vacuum into the FIB chamber and place them onto the stage which is cooled by nitrogen gas, which is led through a liquid nitrogen container and cooled to LN_2 temperature.

14 Coat the grids with organic platinum to avoid damage of the sample by out-of-focus gallium ions. Tilt the stage to 18 ° to allow milling at a shallow angle.

15 Find the eucentric height of the stage, by correlating the SEM and FIB image and moving the stage iteratively.

Citation: Felix Kraus, vtrinkaus , Ruben Fernandez Busnadiego (04/07/2021). Correlative Light-Electron Microscopy of ÃÂ±-synuclein Aggregates in Primary Neurons. https://dx.doi.org/10.17504/protocols.io.btyinpue

16 Open MAPS software at the FIB computer and take an overview image of the EM grid with the SEM beam.

17 Load the SEM image into the MAPS software (on some instruments where MAPS and the FIB can communicate directly, the image will be automatically uploaded into the software). Apply the three-point-method of correlation to the grid (see also Guo et al., Cell, 2018).

18 For the three-point-method click with the right mouse button onto 'Align'. Select three alignment markers and place them on the SEM as well as on the fluorescence image on points that can be easily recognized (e.g. broken carbon or corners of grid bars). Click 'finish' when you are done and the two images will be overlaid.

19

Then find the cells of interest and zoom in with the SEM. Take again a SEM image and correlate that with the fluorescence image (take care to be in eucentric height and now use the 20 x or 40 x image). Use again the three-point-method to make sure you find the correct cell and region of interest (Figure 1 b, Figure 2 a-c). Do that iteratively until fluorescence image and SEM image match perfectly.

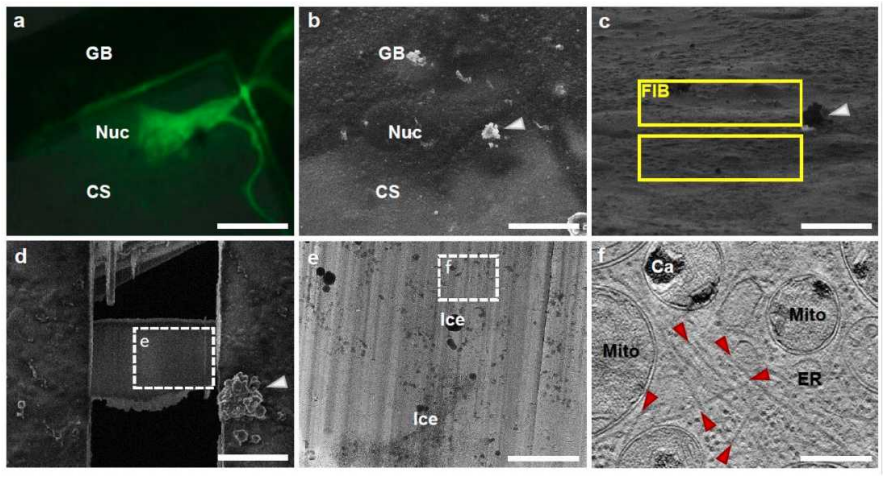

Figure 1: Cryo-light microscopy imaging of GFP fluorescence in a primary neuron grown on the carbon support (CS) of an EM grid. The cell was transduced with GFP-α-Syn at day-in-vitro (DIV) 10 and aggregate formation was seeded at DIV 13. The grid was vitrified at DIV 20. GB: grid bar, Nuc: nucleus. Scale bar: 35 µm. **b**, Correlative scanning electron microscopy imaging of the same cell within the cryo-FIB instrument upon coordinate transformation. A white arrowhead marks a piece of ice crystal contamination that can also be found in panels **c** and **d** as visual reference. Scale bar: 35 µm. **c**, FIB-induced secondary electron image of the same cell. Yellow boxes indicate the regions to be milled away by the FIB during lamella preparation. Scale bar: 20 µm. **d**, Scanning electron microscopy imaging of the same cell upon preparation of a 150 nm-thick electron transparent lamella. The white square marks the region of the lamella shown in **e**. Scale bar: 15 µm. **e**, Low magnification transmission electron microscopy image of the area of the lamella marked in **d**. Ice: ice crystal contamination on the lamella surface. The white square marks the region shown in **f**. Scale bar: 3 µm. **f**, A tomographic slice (thickness 1.4 nm) recorded in the area indicated in **e**. Ca: mitochondrial calcium stores, ER: endoplasmic reticulum, Mito: mitochondrion. Red arrowheads indicate α-Syn fibrils. Scale bar: 300 nm. Taken from Trinkaus et al., BiorXiv, 2020.

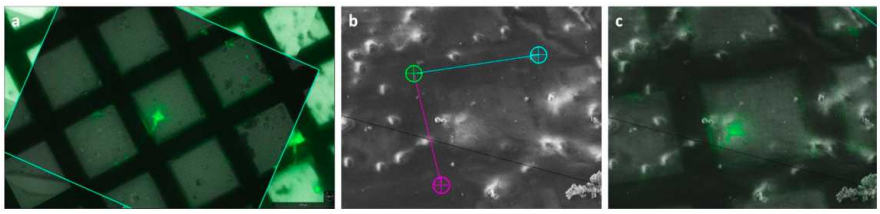

Figure 2: Three-point-method on Tau-YFP neuron containing an aggregate – align the fluorescence image (a) with the SEM image via three points that you can select in the MAPS software (b). Choose striking features like e.g. broken carbon or corners of grid bars. As soon as you finish the alignment, the images will be overlaid (c) and you can inspect if the method worked properly, or if you want to iteratively improve the alignment.

Citation: Felix Kraus, vtrinkaus , Ruben Fernandez Busnadiego (04/07/2021). Correlative Light-Electron Microscopy of ÃÂ±-synuclein Aggregates in Primary Neurons. https://dx.doi.org/10.17504/protocols.io.btyinpue

20

Image the cell in the FIB beam and start with the milling process (Figure 1 c). For milling start with a high current of 0.5 nA at 30 kV and go down to 50 pA for fine milling.

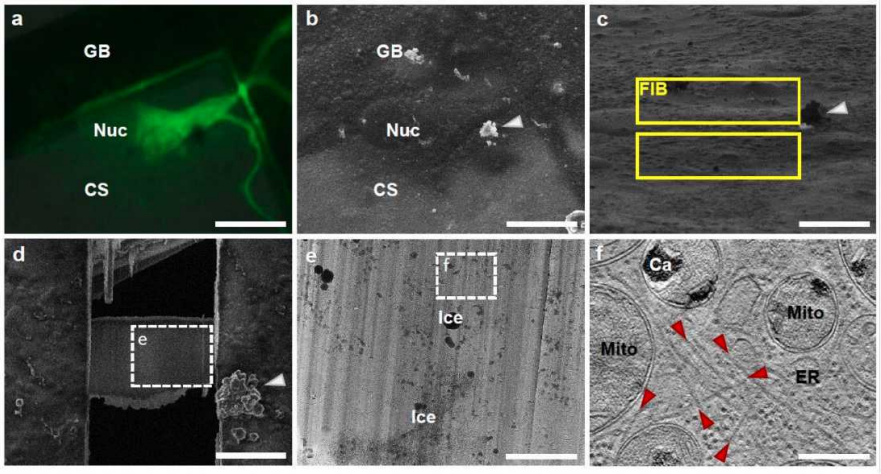

Figure 1: Cryo-light microscopy imaging of GFP fluorescence in a primary neuron grown on the carbon support (CS) of an EM grid. The cell was transduced with GFP-α-Syn at day-in-vitro (DIV) 10 and aggregate formation was seeded at DIV 13. The grid was vitrified at DIV 20. GB: grid bar, Nuc: nucleus. Scale bar: 35 µm. **b**, Correlative scanning electron microscopy imaging of the same cell within the cryo-FIB instrument upon coordinate transformation. A white arrowhead marks a piece of ice crystal contamination that can also be found in panels **c** and **d** as visual reference. Scale bar: 35 µm. **c**, FIB-induced secondary electron image of the same cell. Yellow boxes indicate the regions to be milled away by the FIB during lamella preparation. Scale bar: 20 µm. **d**, Scanning electron microscopy imaging of the same cell upon preparation of a 150 nm-thick electron transparent lamella. The white square marks the region of the lamella shown in **e**. Scale bar: 15 µm. **e**, Low magnification transmission electron microscopy image of the area of the lamella marked in **d**. Ice: ice crystal contamination on the lamella surface. The white square marks the region shown in **f**. Scale bar: 3 µm. **f**, A tomographic slice (thickness 1.4 nm) recorded in the area indicated in **e**. Ca: mitochondrial calcium stores, ER: endoplasmic reticulum, Mito: mitochondrion. Red arrowheads indicate α-Syn fibrils. Scale bar: 300 nm. Taken from Trinkaus et al., BiorXiv, 2020.

21 Mill as long as you get a thin transparent lamella, which is roughly 100 to 150 nm in height. Double check the thickness of the lamellas by imaging it at 3 kV in the SEM. It should be transparent at 3 kV to be sufficiently thin. Every time the stage is moved, repeat the three-point-method, to make sure you still target the correct area.

22 After the milling, transfer the grids out of the FIB under vacuum and store the grids in suitable EM grid boxes.

23 During your next TEM session: Transfer the grids from the boxes into the TEM and acquire tomograms at areas of interest (Figure 1 e and f).

Citation: Felix Kraus, vtrinkaus , Ruben Fernandez Busnadiego (04/07/2021). Correlative Light-Electron Microscopy of ÃÂ±-synuclein Aggregates in Primary Neurons. https://dx.doi.org/10.17504/protocols.io.btyinpue

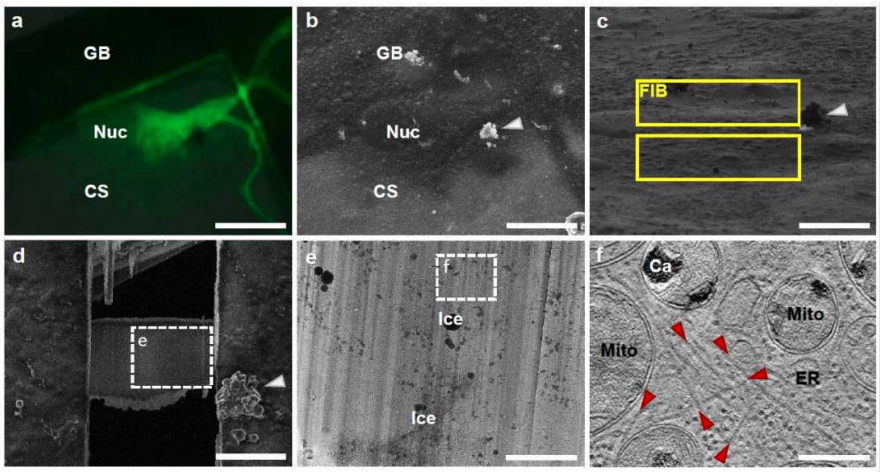

Figure 1: Cryo-light microscopy imaging of GFP fluorescence in a primary neuron grown on the carbon support (CS) of an EM grid. The cell was transduced with GFP-α-Syn at day-in-vitro (DIV) 10 and aggregate formation was seeded at DIV 13. The grid was vitrified at DIV 20. GB: grid bar, Nuc: nucleus. Scale bar: 35 µm. **b**, Correlative scanning electron microscopy imaging of the same cell within the cryo-FIB instrument upon coordinate transformation. A white arrowhead marks a piece of ice crystal contamination that can also be found in panels **c** and **d** as visual reference. Scale bar: 35 µm. **c**, FIB-induced secondary electron image of the same cell. Yellow boxes indicate the regions to be milled away by the FIB during lamella preparation. Scale bar: 20 µm. **d**, Scanning electron microscopy imaging of the same cell upon preparation of a 150 nm-thick electron transparent lamella. The white square marks the region of the lamella shown in **e**. Scale bar: 15 µm. **e**, Low magnification transmission electron microscopy image of the area of the lamella marked in **d**. Ice: ice crystal contamination on the lamella surface. The white square marks the region shown in **f**. Scale bar: 3 µm. **f**, A tomographic slice (thickness 1.4 nm) recorded in the area indicated in **e**. Ca: mitochondrial calcium stores, ER: endoplasmic reticulum, Mito: mitochondrion. Red arrowheads indicate α-Syn fibrils. Scale bar: 300 nm. Taken from Trinkaus et al., BiorXiv, 2020.

Citation: Felix Kraus, vtrinkaus , Ruben Fernandez Busnadiego (04/07/2021). Correlative Light-Electron Microscopy of ÃÂ±-synuclein Aggregates in Primary Neurons. https://dx.doi.org/10.17504/protocols.io.btyinpue