



VERSION 2

MAR 15, 2023

OPEN  ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.yxmvm2kqog3p/v2

Protocol Citation: Felix Kraus 2023. Microscopy-based evaluation of mtKeima flux in hESC-derived Ctrl and FBXO7-/- iNeurons.
protocols.io
<https://dx.doi.org/10.17504/protocols.io.yxmvm2kqog3p/v2>
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Protocol status: Working
We use this protocol and it's working

Created: Mar 15, 2023

Last Modified: Mar 15, 2023

PROTOCOL integer ID:
78870

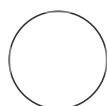
Microscopy-based evaluation of mtKeima flux in hESC-derived Ctrl and FBXO7-/- iNeurons V.2

 In 1 collection

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ABSTRACT

Protocol for the microscopy-based evaluation of mtKeima flux in hESC-derived Ctrl and FBXO7-/- iNeurons

Differentiation of iNeurons

- 1** Day 0: Treat AAVS1-TRE3G-NGN2 cells with Accutase and plate the dissociated cells in matrigel-coated 6-well plates (2x10⁵ cells/well) in ND1 Medium supplemented with Y27632 (10 μ M).
ND1 Medium:
DMEM/F12
N2 (100x) 1x
BDNF 10 ng/ml
NT3 10 ng/ml
NEAA (100X) 1x
Laminin 0.2 μ g/ml
Doxycycline 2 μ g/ml
- 2** Day 1: Replace the medium with ND1 Medium.
- 3** Day 2: Replace the medium with ND2 Medium.
ND2 Medium
Neurobasal medium
B27 (50x) 1x
GlutaMax (100x) 1x
BDNF 10 ng/ml
NT3 10 ng/ml
Doxycycline 2 μ g/ml
- 4** Day 4: Exchange 50% of the medium from each well.
- 5** Day 6: Treat the cells with Accutase and replat the dissociated cells in matrigel-coated
- 6** 6-/12-well glass bottom plates (2-4x10⁵ cells/well for 6 wells) in ND2 Medium.


- 7 Day 8 and thereafter: Exchange 50% of the medium from each well every other day. Doxycycline can be withdrawn on Day.

Live-cell microscopy

- 8 Mount glass bottom plate on Yokogawa CSU-W1 spinning disk confocal on a Nikon Eclipse Ti-E motorized microscope. Ensure that the system is equipped with a Tokai Hit stage top incubator and imaging was performed at 37°C, 5% CO₂ and 95% humidity.
- 9 Take image stacks using a Nikon Plan Apo 60×/1.40 N.A immersion oil objective lens.
- 10 For ratiometric imaging, mtKeimaXL were excited in sequential manner with a Nikon LUN-F XL solid state laser combiner ([laser line – laser power]: 445 - 80mW, 561 – 65 mW]) using a Semrock Di01-T445/515/561 dichroic mirror. Fluorescence emissions were collected through a Chroma ET605/52m [for 445 nm] and a 568 Chroma ET605/52m [for 561 nm], filters, respectively (Chroma Technologies). Consistent laser intensity and exposure times must be maintained for all samples.

Evaluation

- 11 Perform image quantification was in your tool of choice. Here we will use ImageJ/Fiji and custom-written batch-macros (<https://github.com/harperlaboratory/FBX07>)
- 12 Divide raw confocal images of mitochondrial targeted mt-mKeimaXL [ex:561/ex:445], resulting in a ratiometric image of only acidic Keima-puncta.
- 13 Subject resulting signals to background subtraction (rolling kernel size 25, sliding paraboloid) and convert into binary objects.
- 14 Use the "Analyze Particles..." command (pixel size exclusion: 0.5-∞, exclude edge objects) to measure foci-abundance and other morphological parameters.

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- 15** Save results for each image-stack as .csv files, together with the original ratiometric .tiff file for QC purposes.
 - 16** Count number of nuclei for normalization.
 - 17** Plot results in your tool of choice for graphing and statistical analysis.