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The Education, Scholarships, Apprenticeships and Youth Entrepreneurship Programme – EEA Grants 2014-2021

#4 MORPHOLOGICAL ASSESSMENT PROTOCOL USING TRIPLE STAINING (Nucleus – Cytoskeleton – Mitochondrial Networks)

Research Center for Advanced Medicine – Medfuture (UMFIH) can perform different functional *in vitro* tests for evaluation of treatment response on transformed or primary cell lines. More info can be found at: https://medfuture.umfcluj.ro/.

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Cell triple coloration protocol is often used to assess the morphological appearance of the cell in order to gather data about cell function, differentiation, signal response and viability in response to a specific stimuli or treatment.

a. Cultured cells are counted using the Burker chamber or EVE reader slides using TrypanBlue
b. Each chamber slide is labeled to identify the experimental groups in the wells according to the specifics of the project (e.g. control and treatments, treatments at various timepoints)
c. Culture medium and cell suspension are added in the chamber slide. The number of cells may vary depending on the experiment in progress. In the case of the microscopy chamberslides it is recommended to use the appropriate volumes, in a maximum 500 uL final volume/chamber
d. Incubate the cells according to the experimental protocol
e. After 24h (or according to the experimental protocol) add the treatment (if applicable) and incubate for another 24h (or other incubation time according to the experimental protocol)
f. After the incubation steps, wash the sample three times with phosphate buffer (PBS1X)
g. Add the working solution of Mitotracker (prepared according to the protocol from the manufacturer's technical book/ 5 uL of the stock is brought to 2500 uL with buffer from the kit). Frequently, it is used 100 uL Mitotracker and 100 uL culture medium (it is important to respect the ratio v/v=1/1.). Mitotracker must be added prior to cell fixation to bind to metabolically active cells.
h. Incubate for 1 hour at 37°C, protected from light.
i. After incubation, wash the sample three times with phosphate buffer (PBS1X)
j. Add paraformaldehyde 4% (PFA 4%) to fix the cells and incubate at room temperature for 5-10 minutes
k. Wash with PBS1X 3 times
l. Permeabilize the cells with Triton X 0.5% for 5 minutes at room temperature
m. Wash with PBS1X 3 times
n. Add Phalloidin (working solution, prepared according to the manufacturer's protocol and kept at room temperature away from light). Phalloidin is added in a volume of 200 µL (100 mM) and incubated for 30 minutes at room temperature, protected from light (add drop by drop on the slide; a volume of 100 µL can be used on the chamber slide)
o. Wash the slide with PBS1X 3 times

p. Add DAPI working solution of a maximum concentration of 200 nM (100 nM ideal). Add 100 uL DAPI and wait 1 minute. Then add 250-500 µL of PBS1X and wait 1 minute for the DAPI crystals to dissolve. Wash with PBS 1X and prepare the sample mounting steps
q. Traces of PBS1X are absorbed with an absorbent tissue and the slide is prepared for reading
r. When preparing the slide, the protocol may vary depending on the solutions used (glycerol or other preparations that prevent the loss of fluorescence)
s. Morphological assessment is performed using the confocal microscope.

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