NCL Method GTA-12

Autophagic Dysfunction in LLC-PK1 Cells

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Protocol adapted from:

Neun, B. W. and Stern, S. T., Monitoring lysosomal activity in nanoparticle-treated cells, in *Characterization of nanoparticles intended for drug delivery*, S. McNeil, Editor. **2011**, Humana Press. p. 207-212. doi: 10.1007/978-1-60327-198-1_22

Please cite this protocol as:

Stern ST, Neun BW, NCL Method GTA-12: Autophagic Dysfunction in LLC-PK1 Cells.

https://ncl.cancer.gov/resources/assay-cascade-protocols DOI: 10.17917/YPTH-N396

1. Introduction

Lysosomal dysfunction is recognized as a potential toxic mechanism for xenobiotics that can result in various pathological states **(1)**. There is concern that nanoparticles, in particular, may cause lysosomal pathologies, since they are likely to accumulate within lysosomes **(2)**. Lysosomal dysfunction could potentially result from nanoparticle biopersistance, or inhibition of lysosomal enzymes, such as inhibition of phospholipase resulting in phospholipidosis, or inhibition of lysosomal protein degradation resulting in lysosomal overload **(1)**. Nanoparticle exposure has also been shown to cause autophagic dysfunction **(3)**, resulting in increased lysosomal mediated degradation of cellular organelles. Common methods used to characterize autophagic dysfunction include direct morphological assessment via light and electron microscopy, in both cell culture and tissue samples, as well as use of lysosomal dyes **(4, 5)**. The method detailed in this protocol utilizes the latter lysosomal dye staining method, as it is suited to high throughput screening.

2. Principles

This assay is based on the method of Rodriguez-Enriquez *et al.* **(6)**, with modifications being substitution of 48-well plating format with 96-well, substitution of rat primary hepatocytes with LLC-PK1 cells, different lysotracker dye incubation conditions, elimination of the fixation step, and addition of celltracker dye for normalization of cell number. Lysotracker Red DND-99 is a cationic fluorescent dye that preferentially accumulates into the acidic lysosomal compartment. Therefore, the amount of dye taken up by cells in culture can be used as an indicator to lysosome content. Decreases in dye uptake, relative to control, could indicate conditions such as decreased lysosomal stability, or, conversely, increases in dye uptake could be indicative of autophagic dysfunction. As other conditions could result in changes in dye uptake, such as changes in lysosomal pH, treatment-related responses to dye uptake should be further evaluated by morphological assessment, using techniques such as electron microscopy, to confirm lysosomal involvement. Celltracker Green CMFDA, is deacetylated within viable cells to a thiol reactive dye that remains in cytosol, and is used to normalize the lysotracker signal to viable cells.

3. Reagents, Materials, Cell Lines, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1   Reagents

   3.1.1   RPMI-1640 Cell Culture Media (phenol-free) (Hyclone, SH3060501)

   3.1.2   M199 Cell Culture Media (Cambrex, 12-109-F)

   3.1.3   Fetal Bovine Serum (Hyclone, SH30070.03)

   3.1.4   Lysotracker Red DND-99 (Invitrogen, L7528)

   3.1.5   Celltracker Green CMFDA (Invitrogen, C7025)

   3.1.6   Hank's Balanced Salt Solution (with calcium and magnesium) (HBSS) (Invitrogen, 14025)

   3.1.7   Nanoparticle

3.2   Materials

   3.2.1   Costar 96 well flat bottom cell culture plates (Corning, 3598)

3.3   Cell Lines

   3.3.1   LLC-PK1 (porcine kidney cells) (ATCC, CL-101)

3.4   Equipment

   3.4.1   Plate reader (Safire^2 –Tecan or equivalent)

4. Reagent and Control Preparation

4.1   50 nM Lysotracker Red/ 10 µM Celltracker Green Co-stain (Light sensitive!)

   4.1.1.   *QS* 5 µL 1 mM Lysotracker Red stock to 995 µL in phenol-free RPMI-1640 media (Solution **A**).

   4.1.2   *QS* 100 µL **A** to 10 mL in phenol-free RPMI-1640 media to make 50 nM working solution (Solution **B**).

   4.1.3   Add 50 µL DMSO to 50 µg Celltracker Green dye (Solution **C**).

   4.1.4   Add 50 µL **C** to solution **B** to make the co-staining solution.

5. Test sample preparation

    5.1   The highest concentration of nanoparticle tested should be at the limit of solubility. The test sample should be at physiological pH. Neutralization of acidic/basic test samples may be required.

    5.2   Dilute test compound in M199 media, making nine 1:4 dilutions for a total of 10 test concentrations.

6. Experimental Procedure

    6.1   Cell Preparation (or as recommended by supplier)

        6.1.1   Harvest cryopreserved cells from prepared flasks **(limit to 20 passages)**.

        6.1.2   Count cell concentration using a coulter counter or hemocytometer.

        6.1.3   Dilute cells to a density of 1×10^5 cells/mL in M199 (3% FBS) cell culture media.

        6.1.4   Plate 100 µL cells/well as per plate format (see Appendix) for three 96-well plates (4 h sample exposure, 24 h sample exposure, and 48 h sample exposure). The format indicates no cells in rows D and E as they serve as controls.

        6.1.5   Incubate plates for 24 h at 5% CO_2, 37˚C and 95% humidity (cells are grown to approximately 80% confluence; Figure 1).
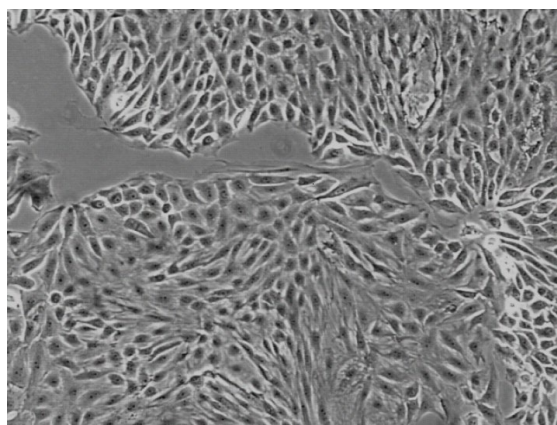

Figure 1. Example of LLC-PK1 Cell Culture Appearance

Image was taken with a phase contrast microscope at 225X magnification. LLC-PK1 cells are approximately 80% confluent at this stage.

6.2 Treatment and staining procedure

6.2.1 Remove media from plate.

6.2.2 Add 100 µL test samples, and positive (HBSS) and media controls as per plating format (see Appendix). Each plate accommodates only one sample. The top half of the plate (Rows A-C) is stained, and the bottom half of the plate (Rows F-H) serves as unstained particle controls to identify assay interference.

6.2.3 Incubate plates for designated time at 5% CO_2, 37˚C and 95% humidity.

6.2.4 Wash plate twice with 200 µL phenol-free RPMI media.

6.2.6 Add 100 µL of 50 nM Lysotracker Red/ 10 µM Celltracker Green co-staining to top half of plate (rows A-D, see Appendix) and phenol-free RPMI-1640 media to the bottom half of the plate (rows E-H, see Appendix), and incubate for 1 h.

6.2.7 Rinse plate twice with 200 µL of phenol-free RPMI-1640 media, add 200 µL phenol-free RPMI-1640 media.

6.2.8 Read fluorescence at (1) 544 nm/590 nm for LysoTracker Red and (2) 492 nm /517 nm, excitation/emission for Cell Tracker Green.

7. Calculations

7.1 % Control Lysotracker Red Fluorescence

$$\%Control\ lysotracker\ red\ fluorscence = \frac{(590nm\ emission\ of\ sample - 590nm\ emission\ of\ sample\ cell\ free\ blank)}{mean\ of\ (590nm\ emission\ of\ control - 590nm\ emission\ of\ control\ cell\ free\ blank)}$$

7.2 % Control Celltracker Green Fluorescence

$$\%Control\ celltracker\ green\ fluorscence = \frac{(517nm\ emission\ of\ sample - 517nm\ emission\ of\ sample\ cell\ free\ blank)}{mean\ of\ (517nm\ emission\ of\ control - 517nm\ emission\ of\ control\ cell\ free\ blank)}$$

7.3 Report Ratio

$$Report\ ratio = \frac{\%\ control\ lysotracker\ red\ fluorescence}{\%\ control\ celltracker\ green\ fluorescence}$$

Mean, SD and % CV should be calculated for each positive control and unknown sample.

8. Acceptance Criteria

    8.1   Fluorescence of the sample dilution wells on the bottom, unstained portion of the plate indicates that the test material may cause assay interference.

    8.2   The starvation positive control wells average should be 150% of media control or greater at 24 h, and % CV should be less than 50%.

    8.3   If the acceptance criteria outlined in 8.2 are not met, the assay should be repeated.

    8.4   The % CV of the sample dilution wells should be less than 50%, or the assay should be repeated.

9. References

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10. Abbreviations

HBSS   Hank's Balanced Salt Solution

CMFDA   5-chloromethylfluorescein diacetate

CV   coefficient of variation

DMSO   dimethyl sulfoxide

FBS   fetal bovine serum

LLC-PK1 cells   renal epithelial cell line, porcine kidney

RPMI   Roswell Park Memorial Institute

QS   *Quantum sufficiat*

SD   standard deviation

11. Appendix

Example of a Plate Format

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cells Media	Cells Sample Dilution 10	Cells Sample Dilution 9	Cells Sample Dilution 8	Cells Sample Dilution 7	Cells Sample Dilution 6	Cells Sample Dilution 5	Cells Sample Dilution 4	Cells Sample Dilution 3	Cells Sample Dilution 2	Cells Sample Dilution 1	Cells HBSS
B	Cells Media	Cells Sample Dilution 10	Cells Sample Dilution 9	Cells Sample Dilution 8	Cells Sample Dilution 7	Cells Sample Dilution 6	Cells Sample Dilution 5	Cells Sample Dilution 4	Cells Sample Dilution 3	Cells Sample Dilution 2	Cells Sample Dilution 1	Cells HBSS
C	Cells Media	Cells Sample Dilution 10	Cells Sample Dilution 9	Cells Sample Dilution 8	Cells Sample Dilution 7	Cells Sample Dilution 6	Cells Sample Dilution 5	Cells Sample Dilution 4	Cells Sample Dilution 3	Cells Sample Dilution 2	Cells Sample Dilution 1	Cells HBSS
D	Media Media	Media Sample Dilution 10	Media Sample Dilution 9	Media Sample Dilution 8	Media Sample Dilution 7	Media Sample Dilution 6	Media Sample Dilution 5	Media Sample Dilution 4	Media Sample Dilution 3	Media Sample Dilution 2	Media Sample Dilution 1	Media HBSS
E	Media Media	Media Sample Dilution 10	Media Sample Dilution 9	Media Sample Dilution 8	Media Sample Dilution 7	Media Sample Dilution 6	Media Sample Dilution 5	Media Sample Dilution 4	Media Sample Dilution 3	Media Sample Dilution 2	Media Sample Dilution 1	Media HBSS
F	Cells Media	Cells Sample Dilution 10	Cells Sample Dilution 9	Cells Sample Dilution 8	Cells Sample Dilution 7	Cells Sample Dilution 6	Cells Sample Dilution 5	Cells Sample Dilution 4	Cells Sample Dilution 3	Cells Sample Dilution 2	Cells Sample Dilution 1	Cells HBSS
G	Cells Media	Cells Sample Dilution 10	Cells Sample Dilution 9	Cells Sample Dilution 8	Cells Sample Dilution 7	Cells Sample Dilution 6	Cells Sample Dilution 5	Cells Sample Dilution 4	Cells Sample Dilution 3	Cells Sample Dilution 2	Cells Sample Dilution 1	Cells HBSS
H	Cells Media	Cells Sample Dilution 10	Cells Sample Dilution 9	Cells Sample Dilution 8	Cells Sample Dilution 7	Cells Sample Dilution 6	Cells Sample Dilution 5	Cells Sample Dilution 4	Cells Sample Dilution 3	Cells Sample Dilution 2	Cells Sample Dilution 1	Cells HBSS