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Intracellular Accumulation of HiLyte-488-A β 42 in DU145 and DU145-TXR Cells

Lauren Ammerman¹¹University of Texas Southwestern Medical Center at Dallas

Lauren Ammerman: PhD Candidate, Molecular and Cellular Biology

1

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Vogel and Wise Group

Lauren Ammerman

University of Texas Southwestern Medical Center at Dallas

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Protocol to facilitate the imaging, and subsequent quantification, of the intracellular accumulation of HiLyte-488-A β 42 in two cell lines - DU145 (paclitaxel sensitive, non-P-gp-overexpressing human cancer), and DU145-TXR (paclitaxel-resistant, P-gp-overexpressing human cancer) cells. Cells are grown on collagen-treated, sterile glass coverslips in complete medium for 24 hours at 37C in a humidified incubator. After the initial growth phase, complete medium is refreshed, and cells are treated with the following - 1) control solutions (DMSO and Ammonium Hydroxide vehicle controls), 2) 1 μ M HiLyte-488-A β 42 and DMSO vehicle control, 3) 1 μ M Tariquidar (a potent P-gp inhibitor) and Ammonium Hydroxide vehicle control, or 4) 1 μ M HiLyte-488-A β 42 and 1 μ M Tariquidar. Cells are incubated with treatments for 16 hours at 37C in a humidified incubator. After the 16 hour incubation, cells are washed, fixed with paraformaldehyde solution, stained with DAPI, washed twice more, and mounted on slides for imaging. Full details, including necessary calculations, reagent specifics, and notes are included in this protocol.

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HiLyte-488-A β 42, DU145-TXR, DU145, Accumulation Assay, Amyloid Beta, amyloid, P-gp, P-glycoprotein, microscopy, ABC transporter, ABCB1, Intracellular accumulation

_____ protocol ,

Taken by L. Ammerman on a confocal microscope - DU145-TXR cells in the presence of both Tariquidar and HiLyte-488-A β 42.

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Materials with Info

1

Reagent/Material	Manufacturer	Details	CAT#
RPML medium	HyClone	RPML 1640 with L-glutamine and phenol red	SH3002701
Collagen I	Corning	Collagen I, High Concentration, Rat Tail	354249
FBS (Fetal Bovine Serum)	Corning	Regular	35-010-CV
24-well cell culture plate	FisherBrand	Sterile Surface Treated Tissue Cultur	FB012929
Trypan Blue Solution	Gibco	0.4% Trypan Blue	15250061
Pen-Strep	Gibco	Penicillin-Streptomycin	15070063
Trypsin	Gibco	Trypsin-EDTA (0.05%), phenol red	25300062
PBS (Phosphate Buffered Saline solution)	HyClone	1X, 0.067M PO4 PBS	SH3025602
Coverslips	FisherBrand	25 mm	12-545-102P
TC20 Automated Cell Counter	BioRad	Requires slides - Cat #1450011	1450102
HiLyte-488-A β 42	Anaspec	Beta - Amyloid (1 - 42), HiLyte™ Fluor 488 - labeled, Human (LOT# 1958003)	AS-60479-01
Paraformaldehyde Solution	Alfa Aesar	4% Paraformaldehyde in PBS	AAJ61899AP
Fluoromount G	Southern Biotech	Water soluble, nonfluorescing mounting medium	OB100-01

Table of Reagents/Materials.

Preparation

1d

2 Prepare Coverslips and 24-well Plate.

notes - we chose 24-well plates because the wells were big enough to fit our circular coverglasses. This experiment could be done with larger plates; volumes/solutions should be scaled accordingly. Procedures should be performed in a Cell Culture Hood.

1. Sterilize circular glass coverslips in UV light overnight, or using method of choice.
2. Obtain a sterile 24 well cell culture plate.
3. Using clean, sterilized tweezers, place one glass coverslip into each well of the 24 well plate.

A	B	C	D	E	F
coverslip		coverslip			
coverslip		coverslip			
	coverslip		coverslip		
	coverslip		coverslip		

Coverslip placement in a 24 well plate.

3 Collagen-Coat Plates.

notes - we utilized a light collagen treatment of our 24-well plates and slides to 'seal' the edges of the coverslip against the bottom of the flask, thereby preventing cells from growing on the wrong side of the coverslip; and to promote adhesion of cells to the coverslips, thereby preventing cells from washing off during step 7.

Follow the Sigma Aldrich protocol for [coating culture ware with Collagen Type I](#) with the following specifications:

1. Add 500 µL of working Type I Collagen Solution to each well using a P1000.
2. *Using a sterile pipette tip, *gently press down* on top of each coverslip to remove any bubbles, and ensure that the coverslip is flush with the bottom of the plate.
3. Incubate at room temperature (~27C) for 10 minutes.
4. Aspirate collagen solution with a P1000.
5. Add 500 µL of PBS to each well
 - if using immediately, replace the lid of the plate and set aside. Aspirate PBS prior to seeding.
 - if using in 1-2 days, leave PBS in, replace the lid, seal the edges with parafilm, refrigerate.

Passage and Seed Cells.

1h

4 Passage Cells.

notes - our DU145 and DU145-TXR cell lines were a generous gift from Dr. Evan Keller, Univ. of Michigan. DU145 cancer cells are non-P-gp-overexpressing, and chemotherapy-sensitive (specifically Paclitaxel). DU145-TXR cells are P-gp overexpressing and paclitaxel-resistant, and are maintained under selection pressure by supplementing the growth medium with 10 nM Paclitaxel (final concentration in flask) during passaging. See PMID [17440963](#) for more information on these cell lines.

Follow the [ATCC protocol for passaging DU145 cells](#) with the following modifications -

1. Use 0.05% Trypsin-EDTA
2. Incubate cells for 3 minutes with 0.05% Trypsin-EDTA at 37C.
3. Add complete RPMI medium (RPMI 1640, 10% Corning FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco)) to neutralize the trypsin.
4. Spin cell suspension, and conditioned media, at 2000 rpm for 4 minutes.
5. Aspirate media gently, to avoid disturbing the pellet.
6. Re-suspend the pellet in 3 mL of new complete RPMI media.
7. Combine a 15 µL sample with 15 µL of 0.4% Trypan Blue solution and count using the TC20 Automated Cell Counter from BioRad.
8. Once 3 counts have been obtained, and an average live count and live % calculated, seed a flask in complete RPMI media to continue the culture, and move onto step 4.

5 Seed Plates

notes - this concentration gave us areas on the coverslip with ~80-95% confluency, after 24 hours growth + 16 hours incubation with treatments (40 hours total). This will need to be optimized/adjusted for your imaging preferences, and for different cell lines.

1. Adjust the concentration of cell suspension to 60,000 cells / mL. 1 mL of cell suspension / well is required. Add a small amount (~1 or 0.5 mL) of conditioned media to reach the final appropriate volume. TC20 cell counter gives the count in cells/mL.

$$(Ci \text{ cells/mL})(Vi) = (60,000 \text{ cells / mL})((1 \text{ mL} \times \# \text{ wells}) + 0.5 \text{ mL extra})$$

Example: $(5.3 \times 10^5 \text{ cells / mL})(Vi) = (60,000 \text{ cells / mL})(7.5 \text{ mL})$

$$Vi = 0.85 \text{ mL cell suspension} + 6.65 \text{ mL complete media}$$

Final = 0.85 mL cell suspension + 1 mL conditioned media + 5.65 mL new complete media.

2. Add 1mL cell suspension to each well of the collagen treated plate using a P1000.
3. Incubate 24 hours at 37C in a humidified incubator with 5% CO₂.

Prepare and Store ABeta Aliquots

30m

6 Preparation of HiLyte-488-Aβ42 for use in Cell Culture Experiments

notes

- Beta - Amyloid (1 - 42), HiLyte™ Fluor 488 - labeled, Human. HiLyte™ Fluor 488 - DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA; mw 4870.5 g/mol
- This is a fluorescent (HiLyte™ Fluor 488)-labeled β-Amyloid peptide, Abs/Em=503/528 nm. Hilyte 488™ Fluor labeled Aβ (1-42) has a brighter intensity than FAM-labeled Aβ (1-42).
- Manufacturer recommends dissolving in a 1% NH₄OH solution, but no longer includes it.
- Ryan et al. 2013 - "Ammonium hydroxide treatment of Abeta produces an aggregate free solution suitable for biophysical and cell culture characterization". PMID 23678397

1. Dissolve 0.1 mg of the HiLyte-488-Aβ42 peptide in 30 μL of 1% (w/v) ammonium hydroxide in sterile water at room temperature (~27C). Vortex and mix thoroughly.
2. Filter the HiLyte-488-Aβ42 solution using a sterile 0.45 μm pore filter and syringe.
3. Once thoroughly dissolved and mixed, add 380 μL of PBS; final NH₄OH concentration is 0.073%.
4. $(30 \mu\text{L})(0.01 \text{ NH}_4\text{OH}) = (410 \mu\text{L})(X \text{ NH}_4\text{OH})$; = 0.00073 NH₄OH, or 0.073% NH₄OH
5. After mixing thoroughly, the 50 μM peptide solution was aliquoted into 50 μL aliquots, and frozen until use.

6. When using in cell culture experiments, the peptide aliquots were thawed and vortexed immediately before usage and dosing, and were not allowed to sit out for any length of time.
7. Prepare a control solution for use in cell culture assays by adding 30 μL of 1% NH_4OH in sterile water to 380 μL of PBS; use this to control for the presence of NH_4OH in assays.

7 Make Compound Solutions (~23 hours post-seeding)

notes:

- working HiLyte-488-A β 42 solution aliquots are thawed and used once; excess is discarded.
- *never re-freeze/re-use an amyloid beta aliquot.*
- Final volume of each well is 500 μL = 450 μL complete RPMI media + two 25 μL treatments.
- Tariquidar is dissolved in DMSO.
- Our protocol accounts for both the presence of DMSO and NH_4OH in the dosage plan.
- final concentration of DMSO in each well is 0.1%; final concentration of NH_4OH in each well is 0.0037%.
- We chose 1 μM Tariquidar (TQR) because this concentration is sufficient to inhibit most P-glycoprotein in the cells; while TQR has been shown to inhibit BCRP at concentrations near 1 μM ,

1. Tariquidar Solution.

$$(1 \times 10^{-3} \text{ M TQR})(V_i) = (1 \times 10^{-6} \text{ M TQR})(20)(125 \mu\text{L})$$

DMSO correction? = 125 μL (0.02) = 2.5 μL ; we are adding 2.5 μL of 1mM TQR in DMSO, no extra DMSO needed.

$$(2.5 \mu\text{L 1mM TQR in DMSO}) + (122.5 \mu\text{L complete RPMI media})$$

Concentration factor = 500 μL total in well / 25 μL treatment = 20; working solution should be 20X final in well.

2. 2% DMSO Complete RPMI Media (controls for DMSO vehicle)

$$(980 \mu\text{L complete RPMI media}) + (20 \mu\text{L DMSO}).$$

3. HiLyte-488-A β 42 Solution (make this last, as close to dosing time as possible)

$$(5 \times 10^{-5} \text{ M HiLyte-488-A}\beta\text{42})(V_i) = (1 \times 10^{-6} \text{ M HiLyte-488-A}\beta\text{42})(20)(125 \mu\text{L})$$

$$\text{DMSO correction?} = 125 \mu\text{L} (0.02) = 2.5 \mu\text{L}$$

(50 μL of 50 μM HiLyte-488-A β 42 in 0.07% NH_4OH in PBS) + (72.5 μL complete RPMI media) + (2.5 μL DMSO)

4. NH_4OH complete RPMI Media (controls for NH_4OH vehicle)

(50 μL of 0.073% NH_4OH in PBS) + (72.5 μL complete RPMI media) + (2.5 μL DMSO).

(50 μL)(0.073% NH_4OH) = (125 μL)(X) = 0.0292% NH_4OH working solution.

(25 μL)(0.0292% NH_4OH) = (500 μL)(X) = 0.0015% NH_4OH final in well.

8 Dose 24 well plates (24 hours post-seeding).

notes - Keep the HiLyte-488-A β 42 aliquot covered with foil and away from light.

1. Gently aspirate the media from the plate.
2. Add 450 μL of complete RPMI media to all wells – gently! - using a P1000. Pipet onto the side of the well.
3. Add 25 μL of 2% DMSO media to “no treatment” and to “A β control”. – 4 wells total
4. Add 25 μL of diluted ammonium hydroxide control solution to “no treatment” and “TQR only” wells.
5. Add 25 μL of TQR solution to “TQR control” and “A β + TQR” wells.
6. Add 25 μL of HiLyte-488-A β 42 solution to the “A β control”, then “A β + TQR wells”.
7. Add ~ 400 μL of PBS to wells surrounding the seeded wells, as an evaporation protection.
8. Cover the plate with foil.
9. Incubate for 16 hours at 37 C in a humidified incubator with 5% CO_2 .

A	B	C	D	E	F
no treatment		A β control			
no treatment		A β control			
	TQR only		A β + TQR		
	TQR only		A β + TQR		

Plate Plan for A β Accumulation Experiment

9 Plate Processing (16 hrs post dosing).

Notes

- Keep plate protected from light if possible.
- Keep PBS and Paraformaldehyde solutions chilled.
- Aspirate/add solutions from the **side** of the well, *not* the top of the well (to avoid sucking up cells).
- Use a long glass aspirator connected to a gentle vacuum. Rinse tip in DI water when moving from fluorescent-containing wells to non-fluorescent, and vice versa.
- Start with wells that do not contain fluorescent compounds.
- Label your slides before starting this step.
- You can also use Molecular Probes™ SlowFade™ Diamond Antifade Mountant with DAPI (Molecular Probes™ S36964) and skip the DAPI stain.

Fixing/DAPI Staining

1. Gently aspirate media from each well.
2. Add 500 µL cold PBS to each well. Cover with foil. Wait 1 minute. Aspirate and discard.
3. Add 4% Paraformaldehyde in PBS to each well. Cover with foil, wait for 20 minutes. Aspirate and discard.
4. Add 500 µL cold PBS to each well, wait 1 minute. Cover. Aspirate and discard.
5. Add 500 µL DAPI solution (1 to 10,000 DAPI stock in PBS) to each well. Cover. Wait 10 min. Aspirate and discard.
6. Add 500 µL cold PBS to each well. Cover with foil. Wait 5 minutes. Aspirate and discard.
7. Add 500 µL cold PBS to each well. Cover with foil. Wait 5 minutes. Aspirate and discard.
8. Add ~300 µL PBS so that slides don't dry out during transfer.

Transferring Coverslips to Microscope Slides

This is by far the trickiest step of the protocol.

1. Sterilize long curved tweezers with ethanol.
2. Sterilize the needle with a bent end using ethanol - we use a small syringe needle, the end (the very tip on the edge) has been flattened and bent to a 90 degree angle with tweezers or pliers.
3. Set out microscope slides.
4. Add ~10 µL of mounting fluid to each slide using a pipette tip – be careful not to make bubbles, and if you do make bubbles, use the side of the pipette tip to wipe them to one side.
5. Using the tweezers and the bent syringe, lift the coverslip out of the plate.
6. Flip it over, and place it on the mounting fluid.
7. Give it a gentle tap with your tweezers to ensure it is flush with the slide (also expels any bubbles).
8. Store slides, right-side-up, in a box protected from light.

9. Parafilm the box to ensure that the slides do not dry out.
10. Image the next day.

Imaging Notes

10 Notes

- The intracellular fluorescence of HiLyte-488-A β 42 was not diffuse throughout the cytoplasm; see our manuscript for images.
- I developed a quantification method to measure the average brightness (in arbitrary units) of a green fluorescent pixel (HiLyte-488-A β 42) in a cell of interest.
- As shown above, two slides (two samples) were performed per treatment; two trials were performed.
- 6 images were taken per slide, yielding 12 images per treatment in each individual trial.
- Quantification was performed on **raw, unedited** CZI files directly from the microscope.
- For more information, please see our manuscript McCormick & Ammerman et al. 2021 (in-review at PLOS One), "[Transport of Alzheimer's Associated Amyloid- \$\beta\$ Catalyzed by P-glycoprotein](https://doi.org/10.1101/2020.10.22.350777)"; BioRxiv <https://doi.org/10.1101/2020.10.22.350777>

Quantification with FIJI

11 If you use this code, please cite:

1. This protocol with the appropriate DOI
2. McCormick & Ammerman et al. 2021 (in-review at PLOS One), "[Transport of Alzheimer's Associated Amyloid- \$\beta\$ Catalyzed by P-glycoprotein](https://doi.org/10.1101/2020.10.22.350777)"; BioRxiv <https://doi.org/10.1101/2020.10.22.350777>

Relevant citations to include

1. FIJI - Schindelin J et al. "[Fiji: an open-source platform for biological-image analysis](https://doi.org/10.1002/jmi.22186)", PMID 22743772
2. Bio-Formats - Linkert M et al. "[Metadata matters: access to image data in the real world](https://doi.org/10.1093/bioinformatics/btu033)", PMID 20513764

```
11.1 // This code opens raw CZI files from the Microscope using the Bioformats
      // plugin
      // The Green Channel images are converted to a binary mask to facilitate
      // quantification
      // Then uses the 'Analyze Particles' function to measure the brightness, size,
      // dimensions of fluorescent areas.

      // Step 1. Open and load into FIJI
      // uses a directory containing raw CZIs
      dir1 = getDirectory("")
      list = getFileList(dir1);

      // takes the list of files and defines essential variables
      for (i=0; i < list.length; i++) {
        path = dir1 + list[i];
        name = File.getName(path);
```

```

dir = File.getParent(path);
print(File.name);
print(name);
name2 = substring(name, 0, lastIndexOf(name, "."));

// on our files, channel 0 is the gfp channel.
name3 = name + " - C=0";

// import the file, we have variables defined, split into 3 channels. uses raw
// czi file.
// must have bio-formats importer plugin
run("Bio-Formats Importer", "open=[" + path + "]" color_mode=Default
split_channels view=Hyperstack");
selectWindow(name + " - C=1");
close();
selectWindow(name + " - C=2");
close();

// create a duplicate gfp channel image; use to create binary mask
run("Duplicate...", "duplicate channels=0");
selectWindow(name + " - C=0-1");

// identify, threshold, and outline areas of green fluorescence
// subtract minor background
run("Subtract Background...", "rolling=10");

// Unsharp mask helps to define edges of fluorescent areas
run("Unsharp Mask...", "radius=1 mask=0.60");

// threshold the fluorescent areas
setAutoThreshold("Default dark");
run("Threshold...");
setThreshold(15, 255);
setOption("BlackBackground", false);

// save the binary mask as a TIF
// green fluorescent areas are black, all else is white.
run("Convert to Mask");
saveAs("Tiff", dir1+list[i]);

// now, load the original raw green channel.
selectWindow(name + " - C=0");
orig = getTitle();
quotedTitle = "" + orig + "";
print(orig);
print(quotedTitle);
selectWindow(name + " - C=0");

```

```

// use binary mask to define areas to measure on the raw channel
// redirect the measurements to the raw green channel
selectWindow(name2 + ".tif");
run("Set Measurements...", "area mean standard min integrated
area_fraction limit display add decimal=3 redirect="+quotedTitle);
run("Analyze Particles...", "display exclude summarize in_situ");
close(name + " - C=0");
close(name2 + ".tif");

}

```

11.2 // Measures the average brightness of the background in the image so that it can be used to perform background subtraction. The overall change (in post-processing) was negligible, but important.

```

// Step2. Run after FIJI_step1_batch_auto_aBeta_analyzer.ijm
// uses a directory containing raw CZIs
dir1 = getDirectory("")
list = getFileList(dir1);

// takes the list of files and defines essential variables
for (i=0; i < list.length; i++) {
    showProgress(i+1, list.length);
    path = dir1 + list[i];
    name = File.getName(path);
    dir = File.getParent(path);
    print(File.name);
    print(name);
    name2 = substring(name, 0, lastIndexOf(name, "."));
    name3 = name + " - C=0";

    // import the file, we have variables defined, split into 3 channels. uses raw
    czi file.
    // close other channels, load and use the raw green channel.

    run("Bio-Formats Importer", "open=[" + path + "]" color_mode=Default
    split_channels view=Hyperstack");
    selectWindow(name + " - C=1");
    close();
    selectWindow(name + " - C=2");
    close();
    selectWindow(name + " - C=0");
    orig = getTitle();
    quotedTitle = "" + orig + "";
    print(orig);
    print(quotedTitle);
    selectWindow(name + " - C=0");
}

```

```
// uses the pre-made binary mask from step1
// creates an inverse selection of previously-identified aB regions
// Measures fluorescence of the "background"; convert to value / micron^2 to
compare to particles.
open(dir1, name2 + ".tif");
selectWindow(name2 + ".tif");
run("Create Selection");
run("Make Inverse");
run("Set Measurements...", "area mean standard min integrated
area_fraction limit display add decimal=3 redirect="+quotedTitle);
run("Measure");
close(name + " - C=0");
close(name2 + ".tif");

}
```