A RAPID PLATFORM TO GENERATE LIPOFUSCIN AND SCREEN THERAPEUTIC DRUGS FOR EFFICACY IN LIPOFUSCIN REMOVAL

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Abstract

Lipofuscin is a brown-yellow, autofluorescent polymeric material that accumulates in a ceroid manner within postmitotic cells during aging. Lipofuscin accumulation impairs proteosome and lysosome pathways critical to cell health and homeostasis. Therefore, the ability to quickly generate lipofuscin in vitro, and identify drugs that mitigate the accumulation or clear lipofuscin would be of great benefit to aging research. Here, we present a platform to quickly create lipofuscin-loaded (but otherwise healthy) cells and screen drugs for efficacy in lipofuscin removal. The combination of leupeptin, iron (III) chloride and hydrogen peroxide generates significant amounts of lipofuscin within cells while eliminating the need for a 40% hyperoxic chamber. Alternative methods which load fibroblasts with "artificial" lipofuscin obtained via UV-peroxidation of mitochondrial fragments are much more labor-intensive. This method is faster (≤10 days) than most protocols to generate lipofuscin and assess its removal, which typically require 2 to 4 weeks or longer to complete.

Key words: *lipofuscin, flow cytometry, Fenton chemistry, autofluorescence, fluorescent microscopy*

1. INTRODUCTION

Lipofuscin accumulation has an inverse relationship with lifespan and is a well-documented hallmark of aging. (Nakano et al., 1995; Terman and Brunk, 2004) Many age-related disease states including Alzheimer's (Moreira et al., 2007), Parkinson's (Meredith et al., 2002), and age-related macular degeneration (Sparrow et al., 2000) show increased lipofuscin accumulation. Some organisms accumulate lipofuscin in a nearly linear manner over time, and therefore their age is determined using methods that quantify lipofuscin levels. (Bluhm and Brey, 2001; Cassidy, 2008) Two primary theories have been proposed for lipofuscin formation: the mitochondrial-lysosomal axis theory of aging and the protease inhibitor model of aging. (Ivy et al., 1996; Brunk and Terman, 2002; Terman et al. 2006; Terman et al. 2010) The former focuses on irreparable oxidative damage caused by oxygen-driven Fenton reactions associated with mitochondrial processes, while the latter espouses inadequate lysosomal proteolysis as a cause of aging. Both theories have significant merit and lend credence to the 'garbage catastrophe' theory of aging, which states that the buildup of recalcitrant nondegradable material within the cell eventually leads to cell senescence or inhibited function. (Terman, 2001) Therefore, there is considerable interest in methods to generate lipofuscin to facilitate research on its biological implications and screen drugs for efficacy in lipofuscin removal. However, the vast majority of published lipofuscin generation methods only take one or the other theory into account. (Ivy et al. 1984; Ivy et al. 1988; Marzabadi et al., 1988; Sohal et al., 1989; Ivy et al. 1996; Terman et al., 1999) In fact, to our knowledge only one published lipofuscin generation protocol to date successfully takes into account both theories by exposing cells to leupeptin (a protease inhibitor) and growing the cells in a 40% oxygen hyperoxic chamber. (Terman and Brunk, 1998) Not surprisingly, this method produces a much greater amount of lipofuscin associated autofluorescence in a shorter time than other published methods which only utilize one of the two aging theories. In this paper, we simplify this method by eliminating the need for a 40% oxygen chamber and replacing it with simpler wet chemistry more commonly available to all labs. Specifically, we use leupeptin to inhibit degradation pathways within the lysosome, and $FeCl_3$ and H_2O_2 to create oxidizing conditions through Fenton chemistry. Ultimately, this method generates significant lipofuscin in a much shorter time (≤ 10 days) than previous approaches (2 -4 weeks). (Nilsson et al., 1997)

2. MATERIALS AND METHODS

This is a streamlined method to generate lipofuscin in vitro and test the efficacy of candidate drugs to remove it. There are three main parts to this protocol. First, the experimental design necessary to yield statistically relevant results is presented. Second, a protocol is provided to generate nonbisretinoid lipofuscin-loaded human fibroblasts and quantify lipofuscin autofluorescence levels using flow cytometry. Finally, microscopy and flow cytometry data are presented as method validation.

The procedure is written for 12-well cell culture plates using human skin fibroblasts. Concentrations were optimized to achieve maximal lipofuscin loading and minimal cell death for a particular cell line and type (Coriell # GM00498). Raising the concentration of any ingredient in Table 1 may result in cell death. Conversely, lowering the concentration will decrease lipofuscin loading. Each cell type has a unique tolerance to oxidative stress and protease inhibition. Therefore, the final concentrations of leupeptin, iron (III) chloride and hydrogen peroxide used to generate lipofuscin may need adjusted if a different cell type is used.

2.1 Materials

- 5 mg Leupeptin hemisulfate (Amresco)
- Iron (III) chloride (Sigma)
- 30% Hydrogen Peroxide solution (BDH)
- Molecular Grade Water (G-Biosciences)
- Sterile 2 mL microcentrifuge tubes (VWR)
- Viable human skin fibroblasts (Coriell Catalog # GM00498)
- TrypLE Express (Life Technologies)
- Phosphate Buffered Saline with Calcium and Magnesium (Lonza)
- Phosphate Buffered Saline without Calcium and Magnesium (Lonza)
- EMEM with EBSS and without L-glutamine (Lonza)
- Fetal Bovine Serum (FBS) (HyClone)
- Glutamax (Life Technologies)
- Tissue culture treated 12-well plates (Corning)
- 5 mL polystyrene round-bottom tubes (BD Falcon)

2.2 Equipment

- Microcentrifuge
- Pipette (P1000) and tips
- Flow Cytometer (FACS Canto II, BD Biosciences)

2.3 Stock Solution Preparation

Prepare the following solutions. Leupeptin and cell culture media should be stored at 4°C and can used throughout the experiment. Iron (III) chloride and hydrogen peroxide solutions should be prepared fresh each time either solution is needed.

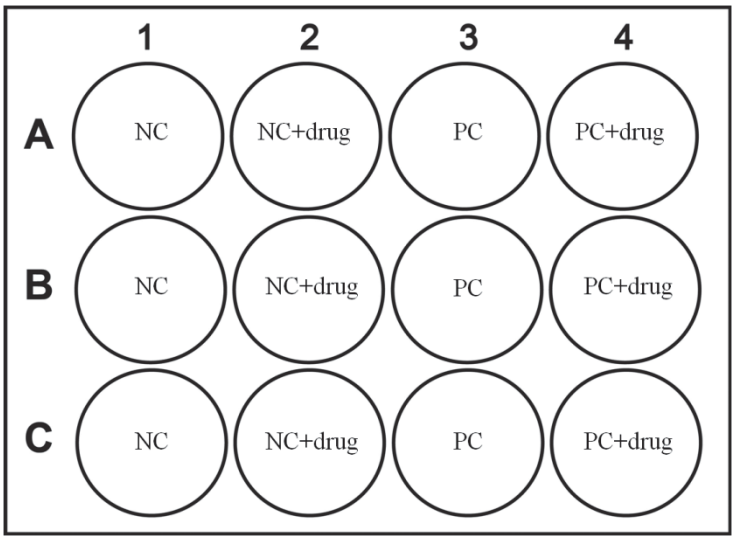
1. Leupeptin stock solution – dissolve 5 mg of leupeptin hemisulfate in 10.13 mL of molecular grade water to make a 1 mM stock solution.

2. Iron (III) chloride solution – dissolve 30.41 mg of $FeCl_3$ in 25 mL of molecular grade water to make a 7.5 mM stock solution.
3. Hydrogen peroxide solution – dissolve 2.84 μL of 30% H_2O_2 in 25 mL of molecular grade water to make a 1 mM stock solution.
4. Cell culture media - EMEM, 10% FBS, 1X Glutamax with the optional addition of antibiotics and antifungal reagents to ensure sterility.

3. PROCEDURE

3.1 Part I (12-well plate design)

Four test conditions are utilized, each performed in triplicate. A description of each test condition and recommended plate layout is given in Figure 1.



	Days →				
Condition	1	.....	5	6	10
NC	media (Step 4) for all 10 days				
NC + Drug	media (Step 4) for days 1 - 5			media (Step 4) + drug for days 6-10	
PC	media (Table 1) for all 10 days				
PC + Drug	media (Table 1) for days 1-5			media (Table 1) + drug for days 6-10	

Figure 1. Recommended 12-well plate layout and graphical illustration of treatment scheme over 10 day experiment. NC is negative control. PC is positive control.

3.2 Part II (creating lipofuscin loaded fibroblasts)

5. The stock solutions prepared in Steps 1-3 are added to cell culture as shown in Table 1. Always ensure the culture media and appropriate volumes of stock solution are well-mixed before addition to cells.

Table 1. Recipe for Lipofuscin Generating Media

Reagent to Add	*Volume (μL) to add per mL*	*Final concentration (μM)*
1 mM Leupeptin	40	40
7.5 mM Iron (III) Chloride	6	45
1 mM Hydrogen Peroxide	10	10
Cell Culture Media (Step 4)	944	N/A

6. Once cells have been plated evenly and all wells are nearing confluence (~ >90%) lipofuscin generation may commence. It is important to commence lipofuscin generation before confluence is reached because exposing cells to oxidizing conditions keeps cell layering at a minimum and helps ensure homogeneous cell exposure to oxidants and leupeptin. Add 1 mL of traditional cell culture media (Step 4) to all wells in plate columns 1 and 2. Add 1 mL of media, prepared using Table 1, to all wells in plate columns 3 and 4.
7. Replace media every 48 hours. Each time prepare a new media solution per Table 1 using the stock solutions created in Steps 1-3. With each media change wash cells with phosphate buffered saline (with calcium and magnesium) to eliminate any residual waste products.
8. On the beginning of day 6 add the drug of interest at the desired concentration to all wells in columns 2 and 4. Be sure to add the drug after every 48 hour media change to ensure cells in columns 2 and 4 are continuously exposed to the drug from days 6 though 10.
9. Continue replacing media and drug on a 48 hour schedule as described in steps 6 - 8.
10. On the end of day 10 prepare the cells for flow cytometry analysis.

3.3 Part III (Flow Cytometry)

11. Pre-warm sufficient volumes of TrypLE Express and PBS (without calcium or magnesium) for 10 minutes prior to use
12. Trypsinize cells by adding 400 μL to each well in a 12-well plate
13. Incubate cells for 3-5 minutes with brief manual agitation to promote cell detachment
14. For each individual well from a 12-well plate, transfer cells to a 2 ml centrifuge tube and dilute with 1.6 mL of pre-warmed PBS (from Step 11).
15. Centrifuge cells at 500 g's for 5 minutes. Remove supernatant, gently resuspend cells in 1.5 mL of pre-warmed PBS (from Step 8) and spin cells a second time at 500 × g for 5 minutes.
16. Remove PBS and resuspend cells in 500-750 μL of PBS (from Step 8)
17. Transfer suspending cells to a 5 mL polystyrene round-bottom tubes (i.e. flow cytometer test tube)
18. Measure autofluorescence intensity. For lipofuscin quantification, a 585/42 nm PE band pass filter is used. In general, for lipofuscin quantification, any filter which measures above 560 nm should be considered sufficient. Gating may be applied strictly to prevent analysis of dead or fragmented cells.

4. METHOD VALIDATION

Broad field fluorescent microscopy is an established qualitative approach to verify the presence of lipofuscin and lipopigment as shown in Figure 2. (Terman and Brunk, 1998; Brunk and Terman, 2002) However, the ability to draw definitive quantitative conclusions is limited. Fluorescent microscopy

images are complicated by the heterogeneous nature of lipofuscin accumulation from cell-to-cell, the tendency of fibroblasts to layer upon each other, as well as the washing out of signal during imaging. Images taken at different exposure times are not quantitatively comparable. (Jung, Höhn et al., 2010) Since it is unknown what compounds within lipofuscin cause autofluorescence, researchers sometimes image lipofuscin using multiple filter sets. (Schnell et al., 1999; Höhn et al., 2010; de Castro et al., 2013; Perše et al. 2013) In general, the most common practice to measure lipofuscin uses a long pass emission filter with a minimum wavelength cutoff around 560-590 nm. (Terman and Brunk, 1998; Terman et al., 1999) Alternatively, a band pass filter in the 560-610 nm range is also commonly used. (Sitte et al., 2001) Autofluorescent material imaged using filter sets below 550 nm are sometimes classified as lipopigment rather than lipofuscin; (Song et al., 2014) though, this distinction is by far not concrete given the unknown nature of lipofuscin as well as the ability of fluorophores to emit across multiple filter sets. In general longer wavelengths are chosen to avoid “background” native fluorescence. (Brunk and Terman, 2002) All cells possess a certain degree of native fluorescence (Figure 2A); due largely to natural lipofuscin as well as the presence of natural conjugated-double bond compounds like flavins and porphyrins. As the data presented in this paper shows, the only difference observed when using a FITC range band pass filter (540/40 nm) versus a TRITC (610/60) or a PE (585/42) is the overall strength of fluorescence (Table 2). No difference in fluorescent localization (Figure 2B,C) or efficacy of the test drug is observed between filter sets, and therefore we make no distinguishment between lipofuscin and lipopigment. Figure 2 clearly shows PC samples (Figure 2B) with greater lipofuscin loading than NC samples (Figure 2A). The variation in lipofuscin levels seen between individual cells, particularly Figure 2A, is typical of lipofuscin accumulation. (Brizzee et al., 2013; Perše et al., 2013; Zareba et al., 2014) Such random stochastic variation is inherent and extrinsic to cell function. Inherent processes include transcription rate, regulatory dynamics and genetic factors, while extrinsic processes arise from fluctuations in the amounts or states of other cellular components. (Elowitz et al., 2002; Munksy et al., 2012; Swain et al., 2002) Combined, these processes promote heterogeneous gene expression between cells, which leads to differential lipofuscin accumulation.

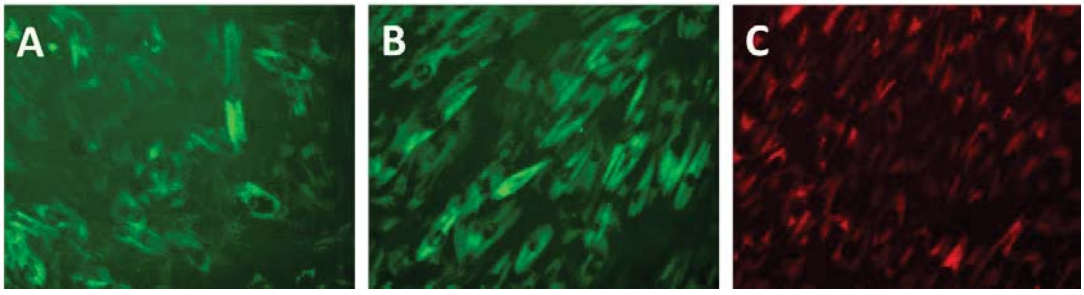


Figure 2. Visualization of Lipofuscin. 200X fluorescent microscopy images of human skin fibroblasts cultured as described in this protocol. A) NC cells with natural lipofuscin (imaged with a FITC 540/40 filter). B) PC cells. C) PC cells (imaged with a TRITC 610/60 filter). Images taken on day 7 of a 10 day experiment.

Confocal microscopy is a useful tool for verifying that autofluorescence is located within small circular puncta, likely the lysosomes, of cells (Figure 3). Confocal images were obtained using an Olympus IX81 microscope under fixed voltage and saturation. Images are reported without changing intensity or contrast.

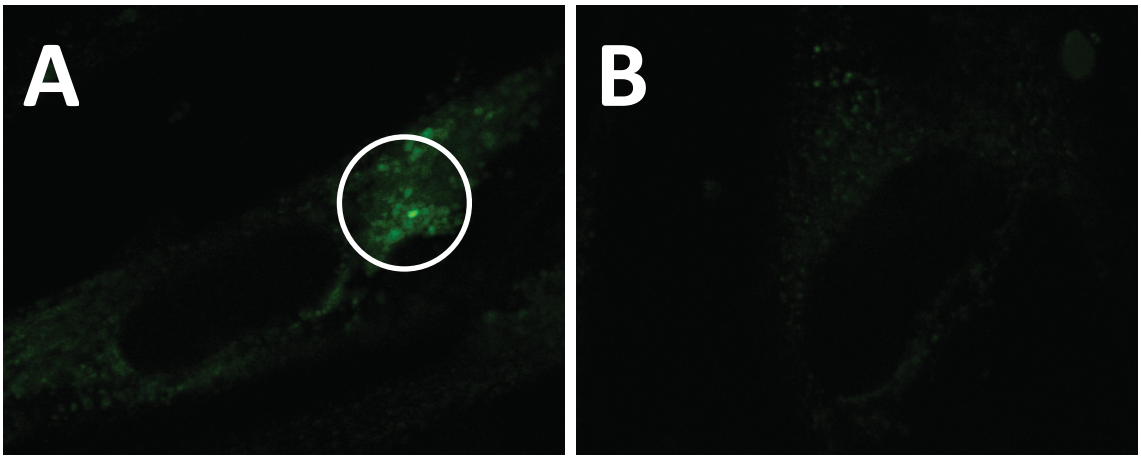


Figure 3. 400X Confocal fluorescent microscopy image of lipofuscin. A) Highlighted area shows autofluorescent material located within small circular puncta, which are indicative of localization to the lysosome. Images show a A) PC sample and B) NC sample after 7 days of lipofuscin generation. Minimal autofluorescence is seen in the NC control sample.

Flow cytometry is a useful high throughput means to quantitatively compare and characterize lipofuscin. Precise autofluorescence values of thousands of individual cells are measured allowing one to compare population shifts in lipofuscin content (Figure 4). Statistical analysis of results obtained for the screening of a candidate drug 'A' is shown in Table 2. In this example, continuous treatment of Drug 'A' from days 6 – 10 as described in this protocol reduced FITC-associated autofluorescence by 33% (p-value = 0.0002) and PE-associated autofluorescence by 27% (p-value = 0.0006).

Table 2. Compiled results for efficacy of sample drug 'A'. Highlighted results show a 27% reduction in lipofuscin-associated autofluorescence when drug 'A' is added from days 6-10. p-value < 0.001 for both FITC and PE filter sets. Both FITC and PE readings are provided and displayed as (FITC/PE).

	Sample 1	*Sample 2*	*Sample 3*	**FITC/PE average**	Std. Deviation
NC	2660/2228	2830/2103	2734/2071	**2741/2134**	85/83
NC + drug	2564/2211	2638/2141	2647/2121	**2616/2158**	46/47
PC	8933/6871	9308/6548	8875/6347	**9039/6589**	235/264
PC + drug	6453/5110	5982/4763	5809/4626	**6081/4833**	333/249

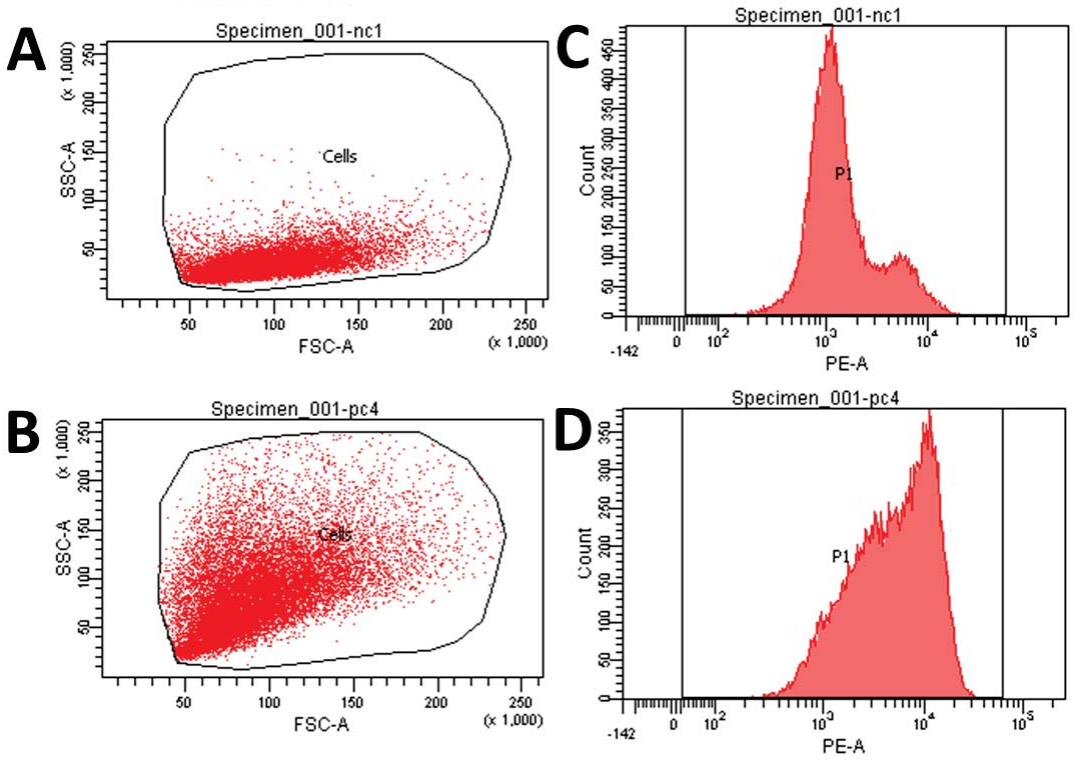


Figure 4. Representative flow cytometry population shifts. Visualization of population shift in size distribution (A and B) and PE fluorescence (C and D) between NC (A and C) samples PC samples (B and D). N > 15,000 cells for both populations.

5. DISCUSSION OF METHOD

An important consideration when choosing this platform is determining whether cross-reactivity exists between the drug being screened and the chemicals being used to generate lipofuscin. Previous studies have demonstrated that protecting cells from oxidizing conditions slows accumulation of lipofuscin. (Thaw et al., 1984) Therefore, if the drug under evaluation neutralizes ferric iron, leupeptin or peroxide before they enter the cell, the assay could yield a false positive. Specifically, rather than removing lipofuscin from within the cell or promoting its removal, the drug would merely prevent further buildup of lipofuscin on days 6 – 10 by attenuating the oxidizing conditions. Therefore, for each successful drug candidate screened, further experiments may be necessary to elucidate the mode of action and ensure that interaction of the oxidants and leupeptin with the test drug is negligible. In most cases, a mixing study incubating the drug of interest with the 40 μM leupeptin, 45 μM $FeCl_3$ and 10 μM H_2O_2 solution for a set time period, followed by mass spectrometry analysis of the drug to assess any chemical changes, will be sufficient. However, the protocols for such experiments are unique to each individual test drug and therefore outside the scope of this manuscript.

6. CONCLUSIONS

Here we provide a fast and facile method to generate lipofuscin-loaded human fibroblasts. Our method uses simple wet chemistry methods which are cost-effective and eliminate the need for a 40% hyperoxic chamber or the biochemical equipment necessary to prepare and characterize UV-peroxidized mitochondrial fragments. We also present a platform-type process to quickly screen drug candidates for efficacy in removing or reducing lipofuscin accumulation, which is of particular interest to the aging and age-related disease research community.

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