

Review

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Review

Lipofuscin and Aging: Testing the Garbage Catastrophe Theory *in cellulo*

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Abstract: Lipofuscin is indigestible garbage that accumulates in the autophagic vesicles and cytosol of post-mitotic cells with age. Drs. Brunk and Terman postulated that lipofuscin accumulation is the main or at least a major driving factor in aging. They even posited that the evolution of memory is the reason why we get lipofuscin at all, as stable synaptic connections must be maintained over time, meaning that the somas of neurons must also remain in the same locale. In other words, they cannot dilute out their garbage over time through cell division. Mechanistically, their position certainly makes sense given that rendering a large percentage of a post-mitotic cell's lysosomes useless must almost certainly negatively affect that cell and the surrounding microenvironment. Here, I explore the possibility that the accumulation of lipofuscin may to some extent exacerbate every other kind of age-related damage. I do not think that lipofuscin removal will reverse/prevent all forms of aging, just the major component facing us currently. In this piece, I will review what is known about lipofuscin accumulation from evolutionary and mechanistic standpoints and discuss a method of removing it from single cells *in vitro*.

Keywords: anti-aging; lipofuscin; *Hydra vulgaris*; TFEB; telomerase; FluidFM

Introduction:

Biological aging is a complex molecular process that takes place over time in all organisms. However, organisms have evolved mechanisms to repair various forms of age-related damage. For example, DNA repair enzymes exist that can fix damage in nuclear DNA. Mitophagy enables the degradation of damaged mitochondria. The immune system, when one is young at least, eliminates at least some proportion of one's senescent cells.

There are many theories about why we age, but one stands out to me as being the most plausible based on the evolutionary and mechanistic evidence. That is the "garbage catastrophe theory of aging." Drs. Brunk and Terman postulated years ago that the problem of aging can essentially be summed up as a "garbage disposal issue [1]." The main idea is that basically old molecules are sometimes damaged in ways that prevent the lysosomes from breaking them down properly, and over time these damaged, old molecules accumulate inside the lysosomes. Eventually, the lysosomes become full of this indigestible garbage, i.e., "lipofuscin", and cannot perform their normal function - then there is a garbage back-up and the cell starts to decline health-wise.

There are two arguments for lipofuscin removal being the most important goal of anti-aging science currently. One is evolutionary and one is mechanistic.

Evolutionary argument:

In nature, there are only a handful of organisms that can be said to be essentially biological immortal. *Hydra vulgaris* (i.e., *magnipapillata*) is one of such organisms, and the reason for this might be that its indigestible garbage is essentially released from its body over time. It has three cell lineages - ectodermal epithelial, interstitial, and endodermal epithelial. All the epithelial cells in its body column are stem cells that continuously divide - displacing cells toward its extremities. The cells at the extremities slough off eventually [2,3]. In terms of the interstitial lineage, the differentiated cells that its stem cells produce are closely associated with epithelial cells and so are continuously displaced as well. This is a convenient way to dispose of lipofuscin - i.e., through dilution and cell

shedding. However, continuous replacement of neurons may not allow for the stable inter-neuronal interactions required for long-term memory [4]. Lobsters continually grow throughout life; their fully differentiated cells express telomerase, allowing them to keep dividing as needed [5]. This includes the cells of their central nervous system, which allows for adult neurogenesis [6]. They also shed their shells periodically. Thus, the same logic appears to apply to them. However, their growth process does not appear to be fast enough to prevent lipofuscin from accumulating over time [7]. Notably, lipofuscin accumulation in eyestalk ganglia [8] is used as a gauge of biological age in lobsters (and myocardial lipofuscin accumulation can be used a marker of chronological age in humans [9]). Lobsters can retain memories, but only for a short time span [10]. Naked mole rats are also very long lived, and it has been shown that they have unusually active autophagic systems [11,12] (in addition to better anti-cancer defenses [13]). Even with better autophagy, naked mole rats still do accumulate lipofuscin in their post-mitotic tissues [14].

It appears as though all animals that age, e.g., flies [15], worms [16,17], lobsters, naked mole rats, mice [18], non-human primates [19], and humans [20] accumulate lipofuscin in their post-mitotic tissues. None of the aforementioned organisms seem to possess any evolutionarily “built-in” ways of exporting the lipofuscin that accumulates in their post-mitotic cells from their bodies, presumably because that would be an unnecessary expenditure of energy in light of procreation.

Export from the post-mitotic cells themselves is possible through exocytosis [21], extracellular vesicle secretion, or secretory autophagy. That part is not too energetically costly. However, from *in vitro* studies, it does not seem as though lipofuscin is exported from post-mitotic cells very often [22]. It also has only rarely been observed *in vivo* [23].

More importantly, when exported, there would ultimately be nowhere for the garbage to go except to be picked up by tissue-resident or circulating phagocytes, which themselves become bloated with lipofuscin. (Transfer of lipofuscin to tissue-resident phagocytes through tunneling nanotubes [TNTs] or partial cell fusion is also theoretically possible.)

Lipofuscin within aged tissue-resident macrophages is perhaps mostly derived from damaged molecules generated by their own, internal metabolic processes - rather than the phagocytosis of extracellular, lipid-saturated debris or efferocytosis in the context of aged, lipofuscin-laden cells [24].

Crucially, I have seen no evidence in the literature that tissue-resident or circulating phagocytes efficiently leave the body through migration to the gastrointestinal tract, urogenital tract, skin, or lungs, except possibly when there are infections in those areas.

Mechanistic argument:

Lipofuscin is broadly a complex amalgam of highly oxidized cross-linked macromolecules, including proteins, lipids, sugars, and metal cations. It varies in composition between species, individuals, cell types, and plausibly even cells of the same type [20,25–27].

While it was originally widely believed that lipofuscin is inert, it may in fact permeabilize or otherwise destabilize lysosomes and promote apoptosis or necrosis [20,28–31]. Even if the damaged molecules are mostly sequestered within lysosomes and are not actively harmful to the cell, the fact that many lysosomes become full of garbage and therefore are almost surely unable to perform their normal functions nearly as well just logically seems as though it must be a major problem for the cell. If a critical threshold is reached in enough cells in a tissue, e.g., the brain, it clearly would be problematic. The cells may try to produce more lysosomes - but will eventually reach capacity.

Along these lines, lipofuscin accumulation decreases the ability of cells to adapt to amino acid starvation [32] and increases their susceptibility to oxidative stress [33]. Increases in the dietary intake of metal cations such as Fe^{2+} , which plays a key role in the formation of lipofuscin, augments lipofuscin accumulation [34–36] and speeds up aging [37]. Manganese acts similarly [38]. Furthermore, artificial lipofuscin loading into human cells results in a significant loss of cellular viability [39,40] - although it is unclear how similar artificial lipofuscin is to real, age-related lipofuscin. Another study showed that the dietary intake of artificial lipofuscin shortens the lifespan of *Drosophila melanogaster* [41].

Dr. Aubrey de Grey postulated that we can co-opt enzymes from soil bacteria and fungi and install them in our cells to degrade our lipofuscin [42]. However, I do not believe this will be feasible, as lipofuscin is quite heterogeneous in terms of composition between species, individuals, cell types, and plausibly even cells of the same type - as mentioned before.

Thus, it would likely require an inordinate number of microbial enzymes to degrade the majority of our lipofuscin, almost all of which would still have to be discovered, evolved, or rationally-designed - and many of them may be toxic to our cells. There are some molecular species that seem to be major contributors to lipofuscin or lipofuscin's toxic effects at least in some cell types, like the fluorophore A2E (retina) [43] and the oxysterol 7-ketocholesterol (throughout the body - especially atherosclerotic plaques and the retina) [44], but we cannot neglect other organs or tissues, or they will fail and we will die anyway. It is worth looking into to see if there are major lipofuscin constituents present across a wide variety of cell types that would be very good targets. But it is unlikely, at least in my mind, that a small number of enzymes will be sufficient to degrade the majority of our lipofuscin deposits.

With regard to short-lived species, like mice and rats, lipofuscin may not have enough time to accumulate to pathological levels before they die of cancer. It is estimated that 50-90% of aged mice die of cancer [45]. Even still, it was shown that in the cerebral cortex neurons of lamina Vb in 630-700-day old rats, lipofuscin occupied 23% of the soma volume [46,47]. This could still certainly have a negative physiological effect. Unsurprisingly, we do see a cognitive decline in mice with age [48]. However, even the oldest mice do not develop age-related neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, or amyotrophic lateral sclerosis [49]. Ostensibly, they simply do not live long enough for sufficient build-up of lipofuscin in their neurons. Other conditions like age-related macular degeneration and sarcopenia do occur in mice, but whether their most severe cases are as bad as the most severe human cases is unclear to me. It is also possible that some mouse tissues accumulate lipofuscin more rapidly than others due to cell type differences in metabolic rates, etc. Some types of human neurons, for example, have not accumulated much lipofuscin by the time others are nearly full [50].

With regard to humans, it has been demonstrated that multiple neuronal cell subtypes become densely packed with lipofuscin granules with age [51-53]. In large motor neurons of centenarians, lipofuscin constitutes up to 75% of total cytoplasmic volume [54]. Other post-mitotic cell types also accumulate substantial amounts of lipofuscin [9,55,56]. Lipofuscin-laden lysosomes are often much larger than typical lysosomes. The typical size of a lysosome in a fed, unaged cell is ~100 nm-500 nm in diameter [57]. In contrast, lipofuscin granules are generally 1-5 microns in diameter [54].

Dysfunctional mitochondria are a hallmark of aging [58]. With lipofuscin accumulation, damaged mitochondria - sometimes with mtDNA mutations, sometimes just with damage to their membranes, proteins, lipids, and DNA - may not be recycled as rapidly and may then start to accumulate. Tau may begin to build up inside neurons due to the garbage back-up, eventually becoming hyper-phosphorylated and forming neurofibrillary tangles. Similarly, β -amyloid that is normally degraded may continue to persist, building to levels that lead to plaque formation. Hopefully, if lipofuscin is removed, plaques that have already formed could regress - in line with Le Chatelier's Principle. In other words, if lipofuscin is cleared and β -amyloid in solution is taken up and autophagocytosed, insoluble plaques may start to dissipate back into solution as well - at which point the β -amyloid in solution would again be taken up and degraded. Transthyretin amyloid could also be another amyloid that forms in the extracellular spaces of our bodies as a result of lipofuscin accumulation in various tissues. Finally, if one could remove the lysosomes from laden macrophages stuck at plaques in artery walls, the plaques may eventually regress, given a healthy diet and sufficient exercise as well [59].

Furthermore, with the extracellular matrix (ECM) not being properly cared for by its resident cells due to lipofuscin accumulation, other damage such as fragmentation, glycation, elastocalcinosis, and cross-linking could rapidly reach pathological levels. After extensive lipofuscin removal, hopefully much of this will damage prove to be reversible. If healthy proteostasis is restored within our parenchymal cells, ECM turnover efficiency could revert to youthful levels [60] - although it may

not be able to reverse all the damage that has accumulated in elderly individuals. Certain cross-links may be cleavable by endogenous, secreted enzymes; after treatment, said cross-link-degrading enzymes could be secreted at the appropriate levels.

Glucosepane is a cross-link that becomes prevalent with age in human tissues that is uncleavable - or at least not cleaved very efficiently/often. Exogenously administered cross-link-breaking enzymes could be helpful [61]. However, there are almost certainly a multitude of indigestible cross-links that accumulate with age. Just like with lipofuscin, an unfeasible number of enzymes might be required to address the problem in this way. Fortunately, even if there are some cross-links that can't be degraded by endogenous enzymes - if the ECM turnover efficiency is restored through extensive lipofuscin removal - molecules bound together by uncleavable cross-links could be excised and endocytosed by tissue-resident cells or phagocytosed by (bioengineered or regular) tissue-resident macrophages.

Senescent cells may start to accumulate if tissue-resident immune cells are rendered inert by lipofuscin accumulation and the non-functionality of the parenchymal cells around them also caused by lipofuscin accumulation, leading to dilapidation of the ECM. Here I refer to irreversibly senescent cells, which have suffered DNA damage and can no longer function properly. However, many cells that show signs of senescence like the senescence-associated secretory phenotype [62] may be *reversibly* senescent. They may have entered into that state due to epigenetic damage brought on by lipofuscin accumulation. Perhaps this epigenetic state would revert back to a youthful state if their lipofuscin can be removed.

Additionally, cancer may be more likely to initiate or progress if many microenvironments throughout the body are corrupted by lipofuscin accumulation. Along those lines, stem cell niche corruption could prevent them from replicating efficiently to replenish tissues.

Blood stem cells from aged individuals can still function normally if they are "rejuvenated" *ex vivo* - and then transplanted into a young niche [63]. It appears as though every compound that I've seen used to "rejuvenate" aged stem cells (e.g., CASIN and rapamycin) actually decreases false lipofuscin in the cells through the stimulation of autophagy [64,65]. From a theoretical perspective, one could imagine that if a stem cell is slowly-dividing, it could build up lipofuscin over time even with some replication. Alternatively, a rapidly-dividing stem cell could be restrained by a niche full of lipofuscin, and then start to accumulate lipofuscin itself. Lipofuscin removal from the stem cells/their niches may make them better able to degrade damaged telomerase components and generate new ones, but it is still possible that a gene vector encoding telomerase could be needed to lengthen the telomeres of our stem cells - preventing them from becoming cancerous or senescent.

A paper published recently in *Nature Aging* that suggests that epigenetic damage is mostly reversible at least [66]. The hallmarks of aging that it related to were mitochondrial dysfunction, nutrient sensing, and stem cell composition. If lipofuscin is removed, damaged mitochondria will be recycled and nutrient sensing processes should go back to normal. Stem cells in this study likely show a younger epigenetic age because they divide frequently and thus dilute out their lipofuscin. That relates to my hypothesis about why Yamanaka factors rejuvenate tissues [67]; I believe it may be because they transiently induce a pluripotent stem cell state. Thus, cells that normally wouldn't divide start to divide transiently, and thereby dilute out their lipofuscin. Sox2, one of the four Yamanaka factors, also initially stimulates autophagy [68] - which decreases "false" lipofuscin. False lipofuscin is intracellular garbage that can be degraded by cellular machinery if said machinery is simply activated more robustly. True lipofuscin is that which is indigestible by cells no matter how they are transcriptionally manipulated - namely the junk that must be removed. Perhaps, however, some age-related epigenetic changes will not revert after lipofuscin removal. If so, the aforementioned rejuvenation technique, known as partial reprogramming, could help to reverse them. However, partial reprogramming may be dangerous; it poses a serious risk of causing cancer through teratoma formation.

Finally, lipofuscin accumulation also explains the downward spiral of functionality that is seen in aging - i.e., the rapid acceleration in decline starting around 60-70 years of age [69]. That is because the decline in autophagy probably increases the rate at which lipofuscin is formed by allowing

aggregates to stay around longer and develop further oxidative damage. Also, lipofuscin accumulation may lead to more free radical production [70], thus accelerating its accumulation as lipofuscin is heavily comprised of oxidatively damaged biomolecules. Furthermore, eventually, when autophagy levels have decreased substantially in many cells in a tissue, the rate of accumulation of other forms of age-related damage probably accelerates as well - and could further accelerate lipofuscin accumulation.

Testing the importance of lipofuscin accumulation in aging *in vitro* now:

TFEB is the master regulator of autophagy (and lysosomal biogenesis) [71]. TFEB overexpression increases lysosomal acidity and boosts lysosomal hydrolase levels in lysosomes. It was shown that TFEB overexpression can shrink lipofuscin deposits - but this work was done in the context of a transgenic mouse model with overproduction of an aggregating protein, which I would define as “false” lipofuscin [72]. Namely, if the protein ceased to be produced and the cells weren’t already completely overwhelmed, it could be degraded over a reasonable amount of time by endogenous lysosomal hydrolases.

TFEB could still be of use for true lipofuscin, however. It also induces the exocytosis of lysosomal contents through fusion of the lysosomal and plasma membranes. One problem with boosting endogenous exocytosis processes is that they may lose potency with age; oxidatively warped lipids from lipofuscin may insert themselves into the membranes of the lysosomes that contain them and prevent efficient fusion with the plasma membrane. The main problem, however, is that the two most well-known inducers of lysosomal exocytosis, Ca^{2+} and TFEB, do not induce a substantial amount of exocytosis - even in youthful cells [73,74].

Notably, a small molecule was shown to induce some lipofuscin exocytosis in monkey retinal pigment epithelial cells, which are important in macular degeneration. However, this small molecule, remofuscin, is a potent and reversible inhibitor of the H^+/K^+ ATPase [75]. Proton pump inhibitors increase lysosomal pH and activate TFEB - so remofuscin’s effects may not be additive with TFEB overexpression.

In order to test the theory that lipofuscin is currently the main issue with regard to age-related disease in a way that would be more definitive, we could utilize a recently developed organelle sampling FluidFM approach [76] to remove lipofuscin-laden lysosomes from single skeletal muscle cells taken from biopsies of elderly humans [77–79]. By doing so, we could observe whether lipofuscin removal reverses the age-related phenotype of these cells. We could also determine how long they live in culture compared to untreated single skeletal muscle cells.

Along these lines, in one study, it was shown that at ~day 25, rat hippocampal neurons *in vitro* have an aged phenotype, including dysfunctional mitochondria, higher reactive oxygen species production, and at least one feature of senescence [80]. It was also shown that defective autophagy was linked to this senescence-like phenotype - and that aged neurons in culture accumulate lipofuscin [81]. Thus, if the post-mitotic cells’ lipofuscin were removed from the cells and new, functional lysosomes were introduced exogenously or generated through TFEB, their aged phenotype may reverse.

Cells from elderly patients may require a more extensive initial session of lipofuscin removal. That is because once the garbage back-up is cleared at the end-point, i.e., the lysosomal level, oxidatively damaged mitochondria would then be mitophagocytosed and cytoplasmic lipofuscin granules would possibly be engulfed by autophagosomes and delivered to new lysosomes. Thus, new lysosomes might be quickly overwhelmed again - and they should be exported as well. Perhaps three rounds of lysosomal removal in quick succession would be appropriate for the first treatment session. The elderly cell may also have autophagosomes that have been present for too long in the cytoplasm. Their outer membranes may have become corrupted by oxidatively warped lipids; thus, they may not even be able to fuse with new lysosomes. They might have to be exported as well.

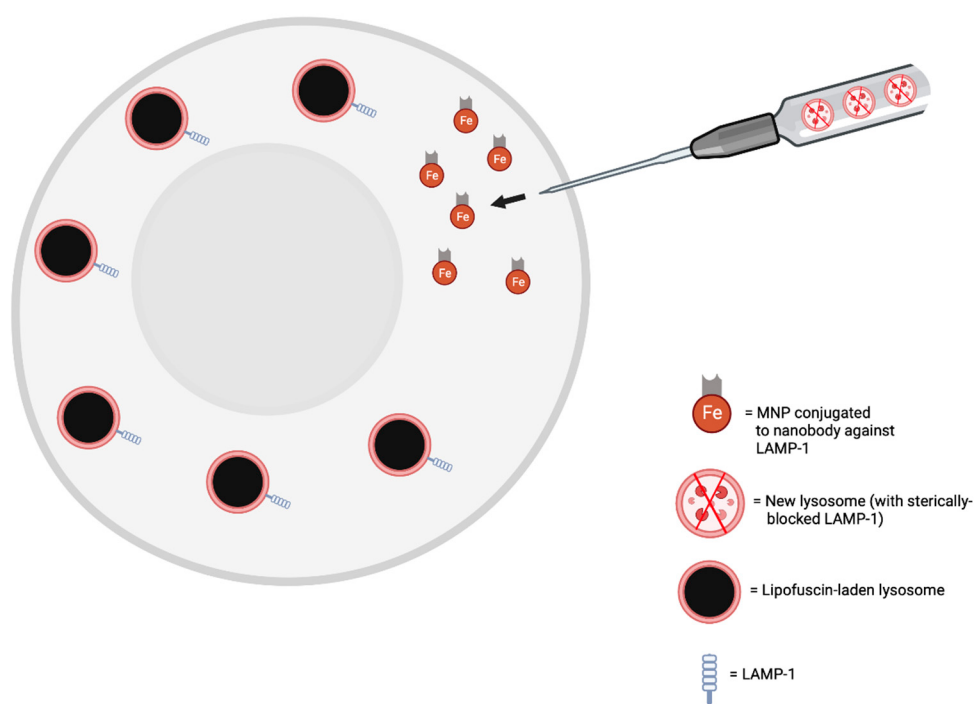
It is important to keep in mind that while primary cell cultures often die after a few days-weeks [82], the cause of death may not be related to normal aging. It may instead be related to infection or

hyperosmolality due to evaporation of the media [83]. These factors should be mitigated or negated if possible. This is perhaps especially tricky when it comes to single-cell manipulations.

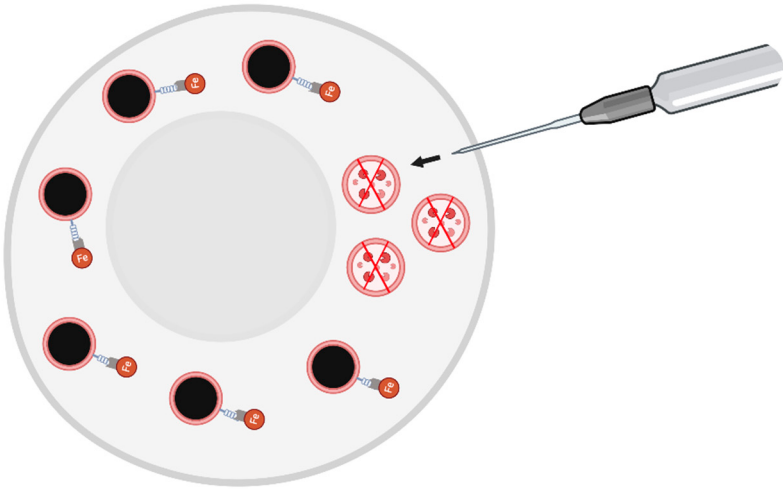
The FluidFM-based approach involves a small needle that penetrates the cell membrane and can inject content into or aspirate content from a single cell [76]. Single skeletal muscle cells from elderly patients could be injected with magnetic nanoparticles (MNPs) conjugated with a nanobodies that target LAMP-1. Then, a magnetic field could be applied to cluster the lysosomes to one small area at the plasma membrane. They would then be removed by the needle. New lysosomes could then be injected to replenish the cell's stores - or perhaps generated via transient, induced TFEB overexpression.

To prevent cell death, one may need to co-inject some number of new lysosomes with the nanobody-conjugated MNPs. The new lysosomes would first be pre-incubated with nanobodies that are not conjugated to MNPs, however - to sterically block the relevant LAMP-1 site.

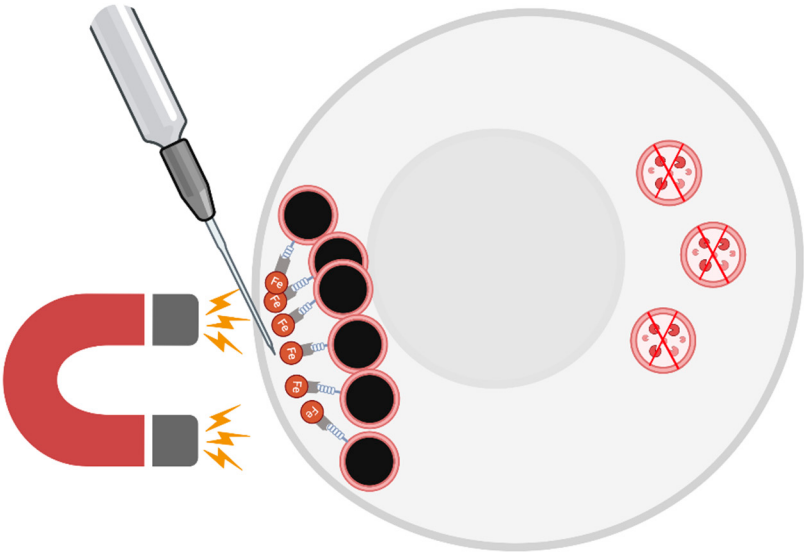
Figure 1: FluidFM for Lipofuscin Removal



(A)



(B)



(C)

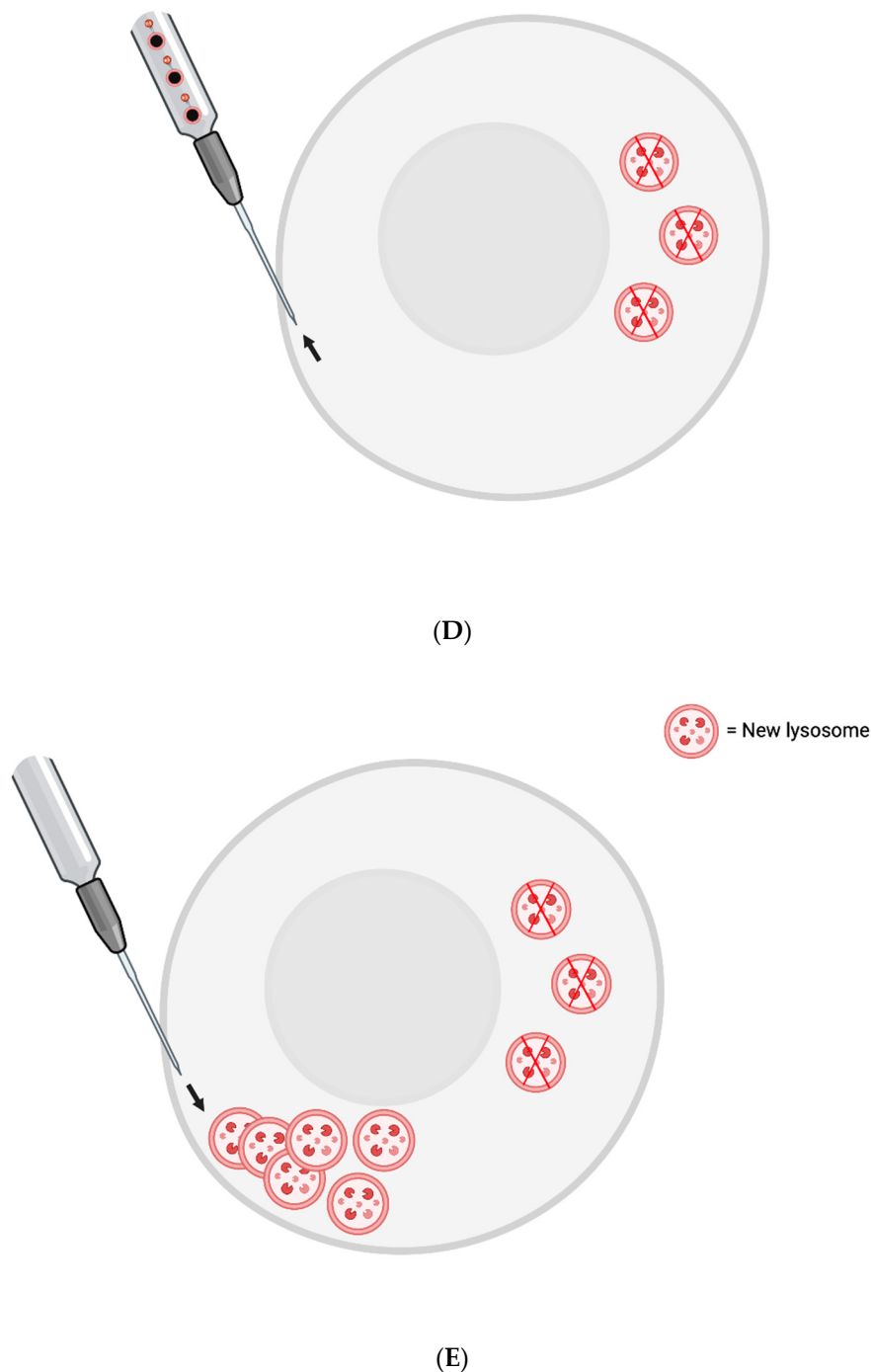


Figure 1. A) Single aged skeletal muscle cells are injected via the FluidFM needle with MNPs conjugated to nanobodies against LAMP-1 and perhaps new lysosomes with sterically-blocked LAMP-1. B) The nanobodies conjugated to MNPs find their LAMP-1 targets on the surface of pre-existing lysosomes. C) Magnetism is used to cluster at least the majority of the pre-existing lysosomes in one area of the cell (i.e., near the needle tip - opposite the region where new lysosomes were injected). D) The needle is used to aspirate the indigestible garbage-filled lysosomes. E) Another set of new lysosomes are injected into the cell where the pre-existing lysosomes were extracted.

Conclusion:

It would be extremely interesting to put the “garbage catastrophe theory of aging” to the test with organelle-sampling FluidFM - using single skeletal muscle cells (in large replicate) from elderly

humans. If *in vitro* testing reveals lipofuscin accumulation to be a critical factor in the aging of such cells, we will have to eliminate it *in situ* or remove it from the body somehow. Through synthetic biology, I believe we can clear lipofuscin from our tissues.

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