Imaging Lysosomes and Secreted Cathepsins

Nina Mohebbi

I. Abstract

Cathepsins, members of the papain family of proteases, have been implicated in the initiation and progression of tumors, cardiovascular disease, and osteoporosis. Due to their involvement in multiple diseases, many studies focus on cathepsins in these disease progressions. Cathepsin inhibitors have also been suggested as treatments for their associated diseases. Unfortunately, due to severe side effects, many inhibitors were terminated at the Phase II clinical testing stage. This suggests not enough is known about the cellular mechanism associated with cathepsins to ensure successful translation of these inhibitors to clinical applications. Due to their localization within lysosomes, we hypothesize that lysosomal activity, movement, and secretion will be tied to levels of cathepsin activity. Fluorescence microscopy with custom image analysis is used to track lysosomes within the cell and biochemical assays are used to assess enzymatic activity. These results are used to evaluate potential correlations between lysosome characteristics, such as size and/or cathepsin localization and the level of cathepsin activity. Results from an immunofluorescence experiment showed cathepsin L present inside of the cell is not exclusive to storage within lysosomes. Results from image analysis also show an increase in lysosome size when cathepsins inhibitor E-64 is added to cells. This could be a result of cargo in lysosomes not being broken down. The long-term implications of this research include providing clinicians and pharmaceutical developers with ways to predict the rate of disease progression on a patient-to-patient basis, and therefore more personally evaluate risk and designate aggressive or more passive treatment options.

II. Introduction

Cathepsins are part of the papain family of proteases and are found within lysosomes, the organelles which contain enzymes and function as the digestive system of the cell, degrading and digest material in the cell [1, 2]. There are 11 identified human cysteine cathepsins: B, C, F, H, K, L V, O, S, W, and Z (or X) known to function in other cellular compartments and extracellular space [3]. Cathepsin activity has been linked to the initiation and progression of tumors, cardiovascular disease, and osteoporosis[4, 5]. Due to their involvement in many diseases cathepsins have been studied for many years as biomarkers for disease progression and subsequently cathepsin inhibitors have been suggested as pharmaceuticals to stem disease progression [6, 7]. The focus of many pharmaceutical producers shifted to cathepsin research after the phase III clinical trial failures of several drug programs focused on matrix metalloproteases (MMPs) in the late 1990s [8]. Between the years of 2004 and 2010 alone, 50 patent applications were filed for potential cathepsin inhibitors. This includes a drug being developed by Merck which currently shows promising results in the Phase III trials for osteoporosis [9]. However, in other cases, despite the promise shown by cathepsin inhibitors in experimental models, many inhibitors were terminated at the Phase II clinical stage due to severe side effects. This result suggests that more information about the cellular mechanism of cathepsins is necessary to translate to clinical applications [8, 9].

One potential source of this mistranslation is in patient-to-patient variability. Over-estimation or under-estimation of mature cathepsin expression and activity in patients could result in improper dosing. Along the same line of study, we hypothesize that variability of intracellular cathepsin as well as secreted cathepsin activity could be studied as a marker for the rate of disease progression in different people [6, 10]. Though studies have been done on factors

such as genetic loci, not many have been done on the cellular mechanism. Understanding the cellular behavior associated with variations in cathepsin gives valuable insight to cell signaling pathways. For this study, we hope to combine florescence microscopy to track lysosomes in the cell with custom image analysis and corroborate enzymatic activity with biochemical assays like zymography. Using these methods a model may be created correlating lysosomal deviations, such as differences in overall diameter, to predict cathepsin activity and corresponding extracellular matrix degradation. In the future this could offer clinicians and pharmaceutical developers with ways to predict disease progression on a patient-to-patient basis. In the future, this knowledge could help evaluate the risk associated with disease progression on an individual basis, and therefore tailor a more specific treatment plan.

III. Literature Review

Interestingly, at their discovery, cysteine cathepsins were considered to be typical lysosomal enzyme scavengers which participate in nonspecific protein degradation. However, according to Fonovic,[4] their proteolytic activity is involved with various pathologies such as cancer, arthritis, and atherosclerosis. Now cathepsins have been identified as a powerful player in many diseases, the question becomes how to approach studying these enzymes, and what are the potential applications. Though many research teams have focused on the correlation between cathepsin activity and disease progression, not enough is known about the cellular mechanisms which are involved with their activities. This study proposes that it is essential to the success of cathepsin research and applications to understand more about the upstream and downstream steps of cathepsin activity, on the cellular level.

As evidence to this, upon discovery cathepsins were thought to be solely lysosomal scavengers; however, this has been thoroughly disproved, and this knowledge of their molecular activity has been essential to most studies done on cathepsins. If cathepsins were not secreted from lysosomes they would not be considered as players in tissue remodeling and invasion, or the other pathologies with which they are associated today. Upon this discovery, further research now suggest that cathepsins are also active in the nucleus. Goulet[11] detected cathepsin L, localized to the nucleus during G1-S transition through an immunofluorescence experiment, which suggested it may in fact play a role in cell cycle progression. This was supported by findings of Santos-Rosa[12] which provided evidence that cathepsins were involved in the cycle of yeast cell differentiation through a clipping of histone tails. Differentiation of a cell is affected by the structure of chromatin in the nucleus, suggesting cathepsin involvement in another aspect of cell growth. This supports the need for molecular research, which then inspired further study and a potential application for the research.

Cathepsins in Diseases

There have been an immense number of research papers published which connect cathepsin activity to disease progression, many of which are concerned with different cancers and their development. Joyce[13] studied cathepsin effect on invasive growth and angiogenesis, which is the process through which new blood vessels form from pre-existing vessels—an essential process to the development of tumors, and found that, cathepsins were upregulated in pancreatic islet tumors through gene expression profiling. Furthermore there developed a correlation between the use of cathepsin inhibitor at different stages of tumorigenesis and impaired tumor functioning. The tumors were impaired by impacting angiogenesis and progenitor. These results that suggest that cathepsins may play a major role in the process or

initiation of tumor growth, through angiogenesis, and invasion. The connection between tumor progression/invasion and cathepsin upregulation has been supported by many papers, most of which now study upstream or downstream activity in the cells. For example, Gocheva[14] was motivated to study cathepsins, because "high levels of cathepsin protease activity are induced in the majority of macrophages in the microenvironment of pancreatic islet cancers, mammary tumors, and lung metastases during malignant progression." One step further, Gocheva concluded that this correlation was specifically attributed to tumor-associated macrophage-supplied cathepsins. Again, a basic discovery about cathepsin activity- that they are upregulated in cancer cells and inhibition leads to impaired angiogenesis led to an in-depth study by Gocheva which suggested that, in macrophages, interleukin-4 (IL-4) may induce cathepsin activity.

Though cancer is one of the most highly researched pathologies which cathepsins are associated with, as mentioned earlier, cathepsins have been implicated in many others. Osteoclasts, which are the cells that resorb bone during remodeling, were studied by Saftig[15]. This study was done on cathepsin-K-deficient mice and found that cathepsin K played a major role, and was essential to the process of bone remodeling. The bone-marrow space of deficient mice displayed excessive trabeculation (creation of bony matrix) which is associated with an osteoporotic phenotype. An osteoporotic phenotype is characterized by the failure of osteoclasts to efficiently resorb bone. When the mice exhibited this, as well as trabeculation in the marrow, this is evidence that osteoclast functioning was considerably impaired. It also suggested that cathepsin K degrades collagen. This was the initial link between bone resorption and cathepsin K, which connects cathepsin K to bone diseases such as osteoporosis.

Application 1: Cathepsins in the Pharmaceutical Industry

This brings us to one of the three biggest applications of cathepsin research: pharmaceuticals. Since the correlation between cathepsin K and bone resorption was made, a few cathepsin K inhibitors have been suggested as osteoporosis treatments, including odanacatib, which recently has passed into phase III clinical trials in humans. Bone[16] published results from clinical trials done with odanacatib after the phase I trial was complete. It focused on postmenopausal women; this is a logical group to study, as postmenopausal women would be the largest demographic affected by osteoporosis. The experiment was carried out over a period of a year, with an extension year, and evaluated the effect of 0, 3, 10, 25, or 50 mg of odanacatib on postmenopausal women. More specifically, they primarily evaluated the percentage change from baseline lumbar spine bone mineral density (all women who participated in the study started with relatively low bone mineral density, between -2 and -3.5) of the women one year later, along with evaluating other biomarkers of skeletal remodeling. The 50 mg dose resulted in a 5.5% increase, whereas the placebo resulted in essentially 0, exactly -0.2%, change in lumbar spine bone mineral density. Elie[7] worked to create a novel cathepsin inhibitor which works with cathepsins B, L, S, and V. The inhibitor was tested in a model of pancreatic islet cancer. Effective cathepsin inhibitors have prospects for anti-cancer therapy. Their novel inhibitor, VBY-825, was found to significantly decrease tumor burden and tumor number. Which leads them to claim that VBY-825 is potentially an effective anti-tumor drug. However, as stated before, there have been many patents filed for potential cathepsin inhibitors and many clinical trials which use these inhibitors. There needs to be more research done on the actual cellular mechanisms involved with cathepsins and their inhibitors before they can be translated effectively into the clinical applications. Until these mechanisms are understood, there will be a discrepancy in experimental and clinical results.

Application 2: Cathepsins in Research

Though quantitatively measuring cathepsin activity is the next step for pharmaceutical and clinical practices, it is already commonly studied in the research setting. Recently, Terasawa[17] published findings that there is a connection between quantitative cathepsin levels and the efficiency of reovirus killing cancer cells. According to Terasawa, cathepsins B and L are connected to the reovirus effectiveness because they play a role in the proteolytic disassembly of the reovirus outer capsid. After examining the data, Terasawa came to the conclusion that cathepsins B and L were the best biomarker for reovirus-mediated cell killing. So interestingly, though upregulated activity of cathepsins may contribute to cancer cell's invasive and metastatic nature due to their role in tissue remodeling, in this specific case, it also allows cancer cells to be more easily targeted and killed by reovirus. They claim that according to their research, activity of cathepsins B and L was the best biomarker for efficacy of reovirus-mediated oncolysis.

Application 3: Cathepsins to Evaluate Disease Progression

One of the most interesting applications for cathepsins may actually combine principles from the first and second application presented here, and result in increasingly personalized medicine. There have been studies that suggest the progression of diseases can be predicted from their cathepsin levels. One of the first was carried out by Troy[6]. This research was done on 99 patients who were already scheduled to undergo colorectal cancer surgery. Biopsies were done on the removed tumors and the cathepsin B and L levels in normal and cancer samples from the same area were evaluated using an enzyme-linked immunosorbent assay (ELISA). There was a significant correlation between cathepsin B and L antigen and activity ratios. Their data showed that, "cathepsin B and L tumour/normal activity ratios were greater than 1 in early stage disease

and there were gradual reductions in cathepsin B (P ¼ 0:02) and L (P ¼ 0:03) activity ratios with advancing tumour stage." What is potentially even more interesting was that the eventual prognosis of patients (ultimate survival) was inversely related to cathepsins B and L activity ratio. Besides suggesting that cathepsins play an essential role in invasion of tumor cells, these results show the potential for cathepsins to serve as biomarkers for disease progression, and therefore personalized dosing of medicine. There is the potential that the prognosis of a disease can be evaluated at discovery, and this can give doctors a clue as to how aggressive the treatment needs to be. This would be especially applicable in the use of cathepsin inhibitors as drugs. If dosing was evaluated by first evaluating a person's personal cathepsin activity levels, these drugs could be more effective, in addition to becoming much safer.

Importance

The effect of these applications can be far-reaching. The attention was turned to studying cathepsins when, as Palermo[8] suggested, a slew of clinical trials involving matrix metalloproteinases failed. Matrix metalloproteinases were targeted for the fact that they were active proteases, but the side effects that accompanied their dosage were more severe. In addition, the inhibitor was less specific than the potential cathepsin inhibitors. We can learn from the failure of matrix metalloproteinases to prevent something similar from happening with cathepsin inhibitors. Therefore, we should use this as an opportunity to add what was learned in the matrix metalloproteinase studies to these more promising drugs. As Palermo suggested, the next step in cathepsins research may be research into personalization. For this, reliable techniques are needed to evaluate cathepsin expression or upregulation in tumor cells within a person. Palermo went on to suggest that this is essential to determining how patients should be treated with cathepsin inhibitors. Patient-to-patient evaluation of dosing could change the way

diseases are treated by decreasing side-effects and increasing efficiency. Therefore, the next step for cathepsin research is to find more efficient ways, meaning ways which do not require biopsy, to evaluate cathepsin actively levels in diseased tissue. The current study will use imaging to study cathepsin activity and interactions. Different protocols will be utilized to evaluate cathepsin size, as well as location, and these methods will be compared. In the future, these attributes may be studied for correlation with disease characteristics and progression.

IV. Results and Discussion

Measuring fluorescently tagged lysosomes. The first task was to use image processing software to efficiently and effectively measure lysosomes in images taken by super-resolution microscopy. Cells were treated with LysoTracker (LifeTechnologies, Carlsbad CA) and then imaged with structured illumination microscopy (SIM.) The first step in the analysis was to size and enumerate the lysosomes within a cell. For this, we utilized ImageJ for its image processing and analysis tools. Outlined below is the protocol:

1. Load image onto ImageJ
2. Convert image from pixles to microns (Image → Properties)
3. Convert image to a binary (Proccess→ Binary → Make Binary)
4. Obtain a list of lysosomes and their corresponding areas (Analyze →Analyze particles)
    a. For this step you must select your size limits for a particle as well as how you want the result image to appear (for our purpose we used outlines)

Next we determined the appropriate size limits to select for particles. Initially, we did this by comparing the output particles to manually counted particles.

From experimentation with different boundary conditions, the conditions which most consistently resulted in lysosome numbers and sizes close to those counted manually was 0.02-5 μm^2.

Cathepsin localization using immunofluorescence. We predicted that cathepsin localization within the cell would be most easily examined using immunofluorescence. Initially we hoped to use three colors and antibody combinations in order to image cathepsins L and B, as well as lysosomes, by using lysosome associated membrane proteins (LAMP.) Once images of these are taken, they can be overlayed to analyze the colocalization. A high amount of colocalization between a cathepsin and lysosomes would mean the cathepsins are located in lysosomes. Low colocalization would mean they are mostly elsewhere. A low amount of colocalization between the two cathepsins would mean that they are contained in different compartments in the cell. The antibodies we hope to use are in *figure 3*. After cells are treated with the appropriate primary and secondary antibodies, they are imaged using confocal microscopy. Below are images taken of cathepsin L and LAMP using their appropriate excitation/emission wavelengths. As the colocalization image clearly shows, there is not a significant amount of colocalization for cathepsin L and LAMP. This led us to believe that cathepsin L is potentially not lysosomal; this could also explain differing reaction to the addition of E-64 to cells. Further research will be required to support these ideas.

IV. Future Work

To continue this project, we hope to utilize a fluorescent substrate, which is selectively cleaved by cathepsin L, in order to examine where cathepsins are active as opposed to only where they are present. We also hope to compare all these results to the results which are found when cells are exposed to a cathepsin inhibitor, E-64. Preliminary results from zymography show that

cathepsin L will decrease and cathepsin S will increase when E-64 is present. Also using the image processing presented in this proposal, cells with E-64 were shown to have larger lysosomes, which may be a result of cargo buildup when not broken down by lysosomes. We hope by adding substrate studies as well as E-64 studies, we will be able to make a robust statement about cathepsin functioning, especially for cathepsin L.

Acknowledgements

I would like to express my very great appreciation to Dr. Christine Payne and Dr. Manu Platt for allowing me to work on this very interesting project and for their absolutely invaluable advice, mentorship, and support. I would also like to offer my special thanks to Dr. Srikant Iyer for being my close advisor and for going above and beyond with his assistance. The advice, discussion, and technical expertise provided by the entire Payne lab was vital to my research and personal growth. In addition, this research greatly benefitted from the support of the President's Undergraduate Research Award. Last, but not least, I wish to acknowledge the unconditional support provided by my close friends and family.

Addendum

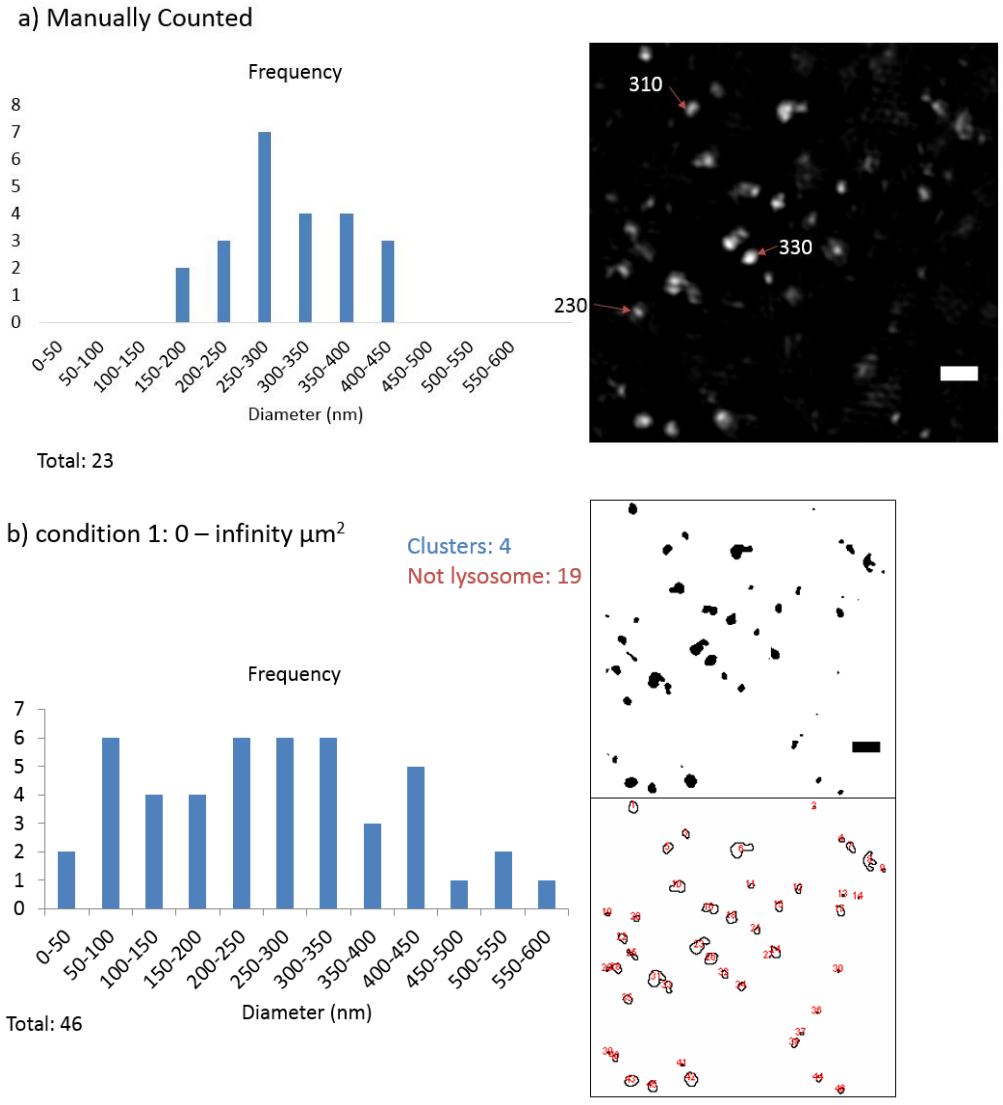

Figure 1 Initial comparison of a small area between manually counted lysosomes and those analyzed by imageJ. *a) Lysosomes were individually counted and their diameter was measured from the original photo taken by SIM. b) Analysis by ImageJ after conversion to binary. Top image- binary without any adjustments. Bottom image- outlines of 'particles' that were counted by the analysis. This is compared to the original image to count 1-objects counted as lysosomes which are not lysosomes ('not lysosome'), and 2- multiple lysosomes which were two close and therefore counted as one large lysosome ('cluster'). Output area was used to solve for diameter and all of them are plotted for comparison.*

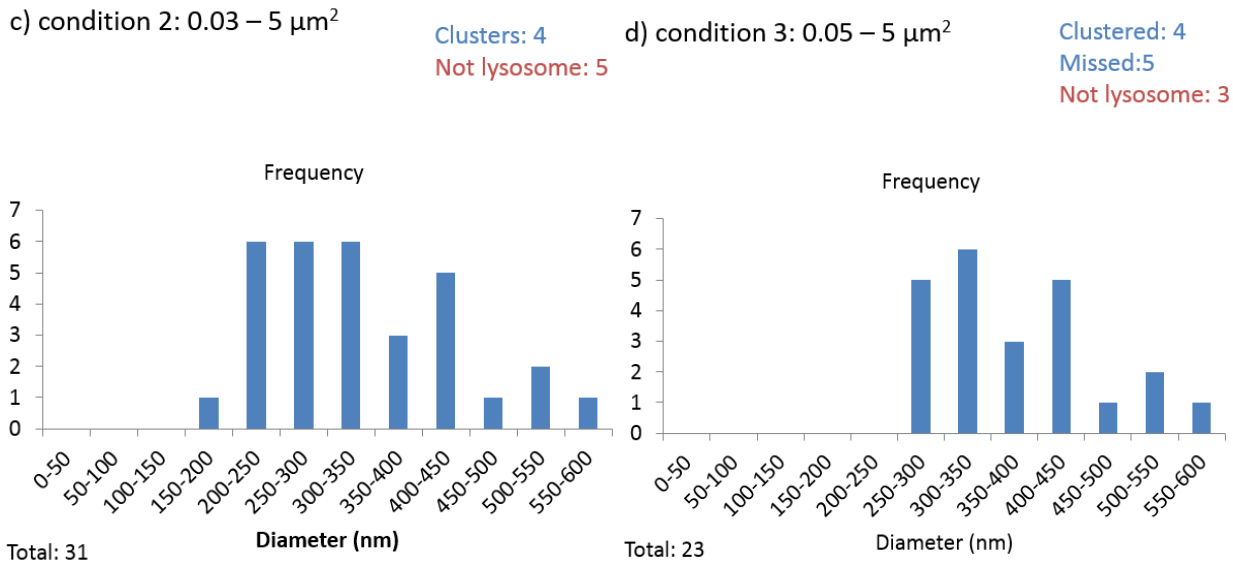

Figure 2 Two examples of trial and error used to determine boundary conditions. *This analysis was repeated using different boundary conditions for the area.*

	Primary AB	Secondary AB	Fluorophore
LAMP	Rabbit α LAMP	Donkey α Rabbit	AF 647
catS	Goat α catS	Donkey α Goat	AF 488
catL	Rat α catL	Donkey α Rat	AF 568

Table 1. Primary and secondary antibodies used. *Careful selection was used to prevent non-specific binding and fluorophore overlapping.*

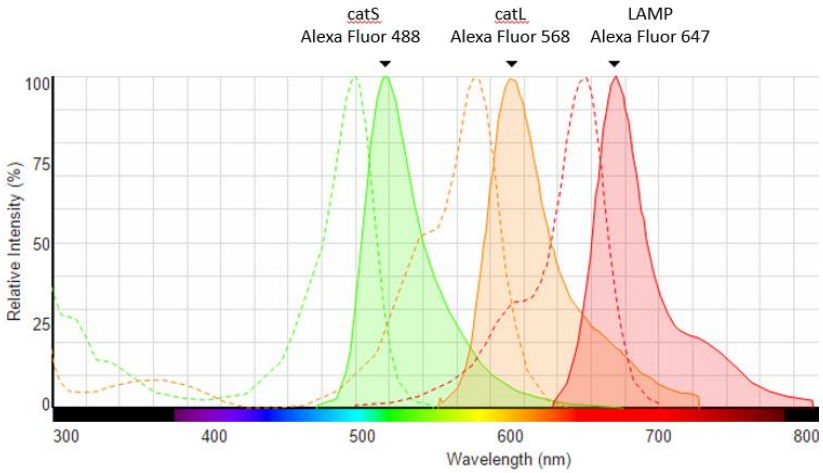

Figure 2 Combination of fluorophores, and antibodies to be used for immunofluoresence experiment. *Designed for minimal overlap between fluorophores and no interaction between antibodies.*

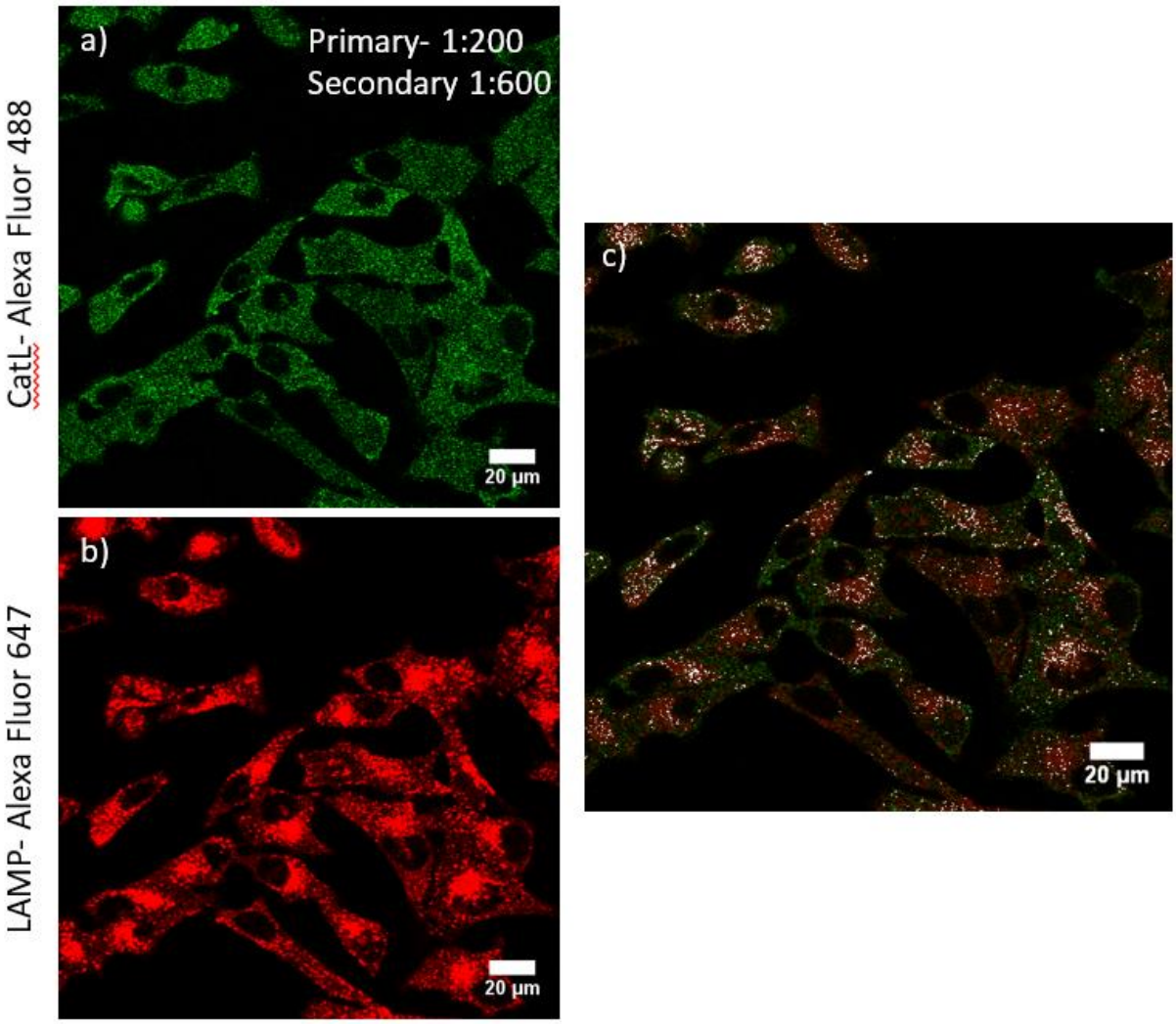

Figure 4. Fluorescent confocal microscopy images. *Antibodies for Cathepsin L and LAMP a) green fluorescence notes the presense of cathepsin L, b) red fluorescece shows where lysosomes are located, c) white spots signify colocalized points where there is green and red fluorescence is present*

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