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Investigations into the use of ALDEFLUOR® as an alternative method to classify leukemia in the soft-shell clam Mya arenaria.
Investigations into the use of ALDEFLUOR® as an alternative method to classify leukemia in the soft-shell clam *Mya arenaria*.

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**Abstract**

The soft-shell clam species *Mya arenaria* is a common model organism in leukemia research. Currently, the typical method for classifying leukemic from non-leukemic specimens is based on visualization of cell concentration and morphology through light microscopy. This approach is highly qualitative, which makes identification of pre-leukemic and semi-leukemic specimens difficult. One more quantitative approach uses flow cytometry to measure levels of aldehyde dehydrogenase (ALDH) expression. In certain human tissues, such as breast tissue, high ALDH expression is a marker for identifying cancer stem cells. The enzyme assay ALDEFLUOR® can actively measure aldehyde dehydrogenase expression in viable cells, but the effectiveness of certain protocol conditions is dependent upon the cell type. This project finds the ideal protocol conditions for the ALDEFLUOR® enzyme assay as an alternative method for classifying *M. arenaria* specimens. It also examines the advantages and disadvantages of both methods, and compares each of them for use in leukemia research.

**Introduction**

Scientists researching the disease leukemia often use the clam species *Mya arenaria* as animal model for human leukemia, due to the high degree of genetic conservation. By studying the molecular mechanisms of leukemia in this model organism, researchers can greater insight
into the nature of the disease without the complications that arise in working with human subjects. However, one of the difficulties that arise in working with clam tissue samples is in the identification of leukemic from non-leukemic subjects. Currently, the typical method for characterization is to microscopically examine a hemolymph sample and visually check for unusual cell morphologies, and extremely high cell concentrations. Unfortunately, this qualitative method can be highly subjective, which can lead to skewed results and poor conclusions. Therefore, there exists a need to have a more quantitative approach for identifying leukemic from non-leukemic organisms.

One popular marker for quantitatively identifying cancerous stem cells in human tissues is isoform one of the enzyme aldehyde dehydrogenase (ALDH1). High levels of ALDH1 expression have been used to identify cancerous cells in human breast, colon, and other epithelial tissues, as well as in hematopoietic tissues like the blood. ALDH enzymes have a very general reaction mechanism, wherein they catalyze the oxidation of an aldehyde group into a carboxylic acid derivative. These enzymes generally have a wide range of specificity, due to the ubiquity of aldehyde functional groups amongst biomolecules. The ALDEFLUOR® assay takes advantage of this property by harnessing ALDH1 to convert a fluorescent dye into its ionized form; this prevents the dye from diffusing back out into the cytoplasm, as the charge prevents it from passively diffusing through the cell membrane. Over time, fluorescent molecules will begin to build up within the cell, giving the cell itself a green fluorescence. This fluorescence can then be measured and quantified through flow cytometry.

Figure 1: The enzyme aldehyde dehydrogenase converts the ALDEFLUOR® from an aldehyde into a carboxylic acid. In its ionized form, this molecule is fluorescent green.
This project proposes to determine the usefulness and efficacy of the Aldefluor enzyme assay as an alternative to the current visualization method. It will also take into account the time and resources required for each method, in addition to comparing method performance. However, before this analysis can be done, a preliminary step must be undertaken. Since ALDEFLUOR® was originally developed for use in human cell lines, the protocol conditions may not necessarily be ideal for use in studying clam tissue. Hence, this project will also test the variables of cell concentration and total incubation time on assay effectiveness in order to find the optimal treatment conditions for clam hemolymph, so that a proper comparison can be made.

**Materials and Methods**

*Hemolymph Extraction*

Clam specimens were obtained in batches of 100 from Spinney Creek Shellfish, a local fishery located in Eliot, Maine. Previous studies of the clam populations in Spinney Creek Lake have shown a high prevalence of leukemic individuals, at roughly 5% of all members. A small portion (<3 mL) of hemolymph was extracted from the heart sinus of each clam using 3 mL and 10 mL syringes. 3-5 drops from each hemolymph sample was placed into a specific well on a 96-well plate.

*Microscopy Visualization*

Each hemolymph sample was individually examined under a compound light microscope and classified as either non-leukemic or leukemic based on cell concentration and overall morphology. In general, samples with smooth, rounded cells of very high cell concentration (approx. ≥ 50% of the total viewing area) were classified as leukemic, and all others were classified as non-leukemic. Any remaining hemolymph in leukemic individuals, as well as from
a few selected non-leukemic individuals, was then also extracted in the manner described above for further sample preparation.

**ALDEFLUOR® Sample Preparation**

A cell count was performed on each hemolymph sample via the Trypan Blue viability assay. Subsequent calculations following these cell counts then allowed for the creation of 1 mL hemolymph cell suspensions in ALDEFLUOR® assay buffer. Suspension concentrations of $1 \times 10^5$ cells/mL, $2 \times 10^5$ cells/mL and $5 \times 10^5$ cells/mL were created from non-leukemic samples, while $5 \times 10^5$ cells/mL, $1 \times 10^6$ cells/mL, and $2 \times 10^6$ cells/mL suspensions were created for leukemic samples.

Each cell suspension was treated with $0.5 \mu$L of the activated ALDEFLUOR® reagent, Bodipy-aminoaetaldehyde (BAAA). 0.5 mL of this suspension was then immediately transferred to a reaction vessel containing $0.5 \mu$L Diethylaminobenzaldehyde (DEAB), to create the corresponding control samples. All samples were incubated in a water bath at 37°C for intervals of 15, 30, 45, 60 or 75 minutes.

Following incubation, cell suspensions were placed in a microcentrifuge where they were spun at 300 x g for 5 minutes at 4°C. The supernatant of each sample was removed and discarded, and the corresponding cell pellet was resuspended in 0.5 mL of ice-cold ALDEFLUOR® assay buffer.

**Flow Cytometry & Data Analysis**

Samples were run through a FACSCalibur flow cytometer at a rate of 60.0 μL/min. Three plot types were generated using the Cyflogic software for each sample type, Forward Scatter (FSC) vs. Side Scatter (SSC), Fluorescent Channel 1 (FL-1) vs. SSC, and SSC vs. Fluorescent Channel
2 (FL-2). The machine was recalibrated for each control/test sample pair by adjusting the voltages and gains so that data points from the control were centered within the FSC vs. SSC dot plot. The machine settings were not altered for the corresponding test sample within each sample pair. At least three data files were collected for each cell suspension, and 10,000 events were recorded for each file. Averages of these data files were then used to generate density plots, 3-D plots, and overlay histograms post-experiment using the Cyflogic software.

**Results**

The traditional visualization method, while simple in theory, has one major drawback in its actual implementation. Leukemia is not a disease which presents itself immediately overnight; it moves instead in a more progressive manner, as the organism transitions slowly to later stages of the disease. For the researcher sifting through large quantities of clams in an attempt to separate the afflicted from the unafflicted, subtle changes in cell morphology may go unnoticed, and organisms in the early-stages of the disease may fall by the wayside. Furthermore, a more inexperienced researcher has a greater potential of making mistakes, and thus may generate a higher number of false positive identifications. Figure 2 below helps to highlight the progression of the disease by displaying the dramatic increase in cell concentration and subtle changes in cell morphology that take place in the hemolymph. Each photograph is representative of a clam in separate stages or non-stages of the disease.
Figure 2:

A: Non-leukemic, displays some cell clumping, and spike-like morphology.
B: Non-leukemic, without clumping.
C: Semi-leukemic, with clumping, smoother morphology.
D: Semi-leukemic, without clumping.
E: Fully-leukemic, without clumping, smooth morphology.
A flow cytometer harnesses the principles of light scattering in order to give information about the contents of a particular solution. When a small portion of the ALDEFLUOR® cell suspension is taken into the machine, it surrounds the particulate matter in a type of sheath fluid before passing this bundle in front of a multi-colored laser. This causes the laser to be slightly deflected from its original position, and the angle of deflection as well as the degree of which the light is scattered is measured by the machine through several different channels. In particular, the FL-1 channel of the FACSCalibur machine is useful for detecting scattering based off of green fluorescence.

When ALDEFLUOR® assay samples are run through the flow cytometer, the results of the laser scattering data in the machine are recorded by the software program Cyflogic. The data is primarily organized through dot plots, and the arrangement of the dots will give the operator information about the generalized characteristics of the cell. A FSC vs. SSC plot will tell one about the sizes and common shapes in a population of cells; the FL-1 vs. SSC plots will tell one about the overall green fluorescence produced by the population of cells, with respect to size, and an SSC vs. FL-2 plot, when used in conjunction with propidium iodide, can give information about the viability of the cells being studied. Since the use of the Aldefluor assay is dependent upon cells being viable at the time of the enzymatic reaction, propidium iodide was not included as part of the sample preparation procedure. SSC vs. FL-2 plots were still included so as to provide more information about fluorescent behavior, as well as to aid in the initial adjustment of laser settings for each sample. Figure 3 below provides an example of the collected data as it is arranged in dot plots. Colors and population labels were added at a later stage by the user as part of the final data analysis.
Figure 3: Averaged laser scattering data taken with a FACSCalibur flow cytometer for leukemic samples incubated for 30 min. at $2 \times 10^6$ cells/mL. Purple and green dots indicate correspondence to populations 1 and 2, respectively. Black dots indicate one located outside of border of the selected regions.

From here, the data from the FL-1 vs. SSC plots can be summarized in terms of an overlay histogram. This type of graph allows for direct comparison of the overall fluorescence between control and test samples for a particular reaction pair. Tables 1 and 2 below organize the histogram data according to the treatment condition variables. For each plot, the x-axis on the histogram corresponds to the amount of scattering the FL-1 laser received due to cell fluorescence, while the y-axis indicates the number of cells which scattered the FL-1 laser at a particular amount. Shaded regions outlined in blue are for the control sample, while regions outlined in green are for the corresponding experimental samples. The amount of overlap between regions is used to judge the overall effectiveness of the test. In the ideal case where the test is highly effective, one would see minimal to no overlap between the histograms of the control and experimental samples.
Table 1: Each of the cells in the table above displays a histogram overlay plot for a specific set of treatment conditions on samples from non-leukemic clams. For example, the graph in the third row and second column represents data from samples incubated for 45 min. at a cell concentration of $2 \times 10^5$ cells/mL.
Table 2: Each of the cells in the table above displays a histogram overlay plot for a specific set of treatment conditions on samples from **leukemic** clams. For example, the graph in the second row and third column represents data from samples incubated for 30 min. at a cell concentration of $2 \times 10^6$ cells/mL.
Discussion

The first observation that one makes from both the scattering data and the histogram overlays is the existence of two distinct sub-populations of cells with markedly different levels of fluorescence. This would indicate that there are cells which have significantly higher levels of ALDH expression than others within the same homogenous sampling. Using microscopy alone, these cells are indistinguishable from one another, and so the prevalence of the dual sub-populations comes as a surprise. Even more surprising is the presence of these dual sub-populations in both leukemic and non-leukemic samples.

These sub-populations take on a variety of shapes in the histogram overlays. The table below provides a generalized description of these shapes as the rest of this paper will refer to the names given below for ease of discussion.

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<th>Table 3:</th>
<th>Generalized Shape</th>
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<td>This table provides a list of the four most common shapes observed in the histogram overlay data.</td>
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Generally speaking, one sees a trend amongst the non-leukemic samples to have a greater overlap between the two-sub-populations, but this does not hold true in all cases. For example, control samples at $1 \times 10^5$ cells/mL and $2 \times 10^5$ cells/mL, incubated for 60 and 75 minutes, all display strong doublet spreads, in comparison to the rest of their non-leukemic control counterparts. The reverse holds true for the leukemic samples; the majority of histograms for both test and control populations display a strong doublet spread, with some samples obtaining complete separation. However, four sets of conditions generated a stretched singlet formation, so this pattern cannot be used to exclusively identify a leukemic clam. On the whole, there does not appear to be any kind of pattern from the histogram data which allows for the complete differentiation of leukemic from non-leukemic samples.

Another very interesting trend arises when one starts comparing test samples to their respective controls. For many tests, there is a leftward shift of the histogram for one, but not the other sub-population. Strictly speaking, it should be impossible for this result to occur, as it would imply that the control samples, which contain the inhibited form of the enzyme, would have higher expression levels than the test samples, which contain the uninhibited enzyme. This would seem to indicate that our control has failed in some manner, as a null result (i.e. one where the change in ALDH expression between test and control is minimized), would occur when the test histogram completely overlapped that of the control, or when the right-handed shift was small enough as to be insignificant. Non-leukemic samples incubated for 45 minutes at $5 \times 10^5$ cells/mL provide a good example of what be typically expected for a null result. If the left-ward shift had occurred to both of the sub-populations, this strange occurrence could have been attributed to experimenter error, and the trial would have been repeated. Intriguingly, however,
the leftward shift only occurs with one of the sub-populations, namely the left-handed one. This population scatters less light than the other, and is overall the less fluorescent of the two.

Furthermore, we can even observe this phenomenon in samples which display a stretched singlet pattern. By breaking the two sub-populations apart and examining their histograms individually, we can easily see how the leftward shift affects only the larger, less fluorescent population. Figure 4 below demonstrates this with an example from both leukemic and non-leukemic sources.

One potential explanation for this phenomenon depends upon the concentration on the ALDEFLUOR® reagent added at the beginning of the assay. It is possible that the smaller, more fluorescent sub-population has the phenotype ALDH-bright, while the larger, less fluorescent population has the phenotype ALDH-low. In both cases, the sub-populations have active ALDH enzymes, but the ALDH-bright cells have much higher levels of enzymatic activity. This kind of phenotypic differentiation has been documented before in other tissue types compatible with ALDEFLUOR®. Changes in amino acid sequencing or post-translational modifications may help account for the differences in phenotype. It is also possible that the ALDH-bright cells may contain special transporters on their plasma membranes which allow for the increased uptake of reagent into those cells. In any case, what we would observe is a more rapid use of the reagent by the ALDH-bright population, which in turn could leave less reagent available for use by the ALDH-low population. Due to the lower concentration of reagent available for the reaction, the enzyme activity becomes decreased. By inhibiting ALDH in the control samples, more of the reagent becomes available for use by both populations, and the “stealing effect” is silenced.
Figure 4: The histograms on the right are based off of the selected regions from the original scatterplots, which are not shown here. Population 1, in each case, refers to the larger region on the left-hand side which scattered less light overall, while Population 2 refers to the smaller region with greater scattering. The samples in the top half of the diagram were taken from a leukemic clam, while those in the bottom half of the diagram were taken from a non-leukemic clam. Both samples were incubated for 75 min. at a concentration of $5 \times 10^5$ cells/mL.
One way to test the stealing effect hypothesis would be to increase the initial concentration of ALDEFLUOR® reagent when it is added at the beginning of the reaction process, and see if it has any measurable effect on the degree of the leftward shifts. Figure 5 below displays the results of increasing the ALDEFLUOR® reagent concentration to 15 uL for leukemic samples incubated for 30 minutes with a cell concentration of $2 \times 10^6$ cells/mL.

Immediately, one observes the highly intriguing result of a third sub-population for the test sample scattering data. Equally intriguing is the absence of any clearly defined second sub-population for the control scattering data. This result would seem to imply that the factors affecting fluorescence, and thus ALDH activity, are much more complicated than first thought. The histogram overlay also provides little in the way of explanation for this phenomenon. The control sample takes on the form of a weak right-handed doublet, while the test sample displays a different shape entirely. It most closely resembles that of a strong doublet, with the height of the secondary hill being greatly reduced; however, it also has characteristics of a stretched singlet. Due to the radical difference in shape between control and test histograms, it is difficult to make a conclusion regarding the overall shift of the populations. Clearly, more tests are in order to fully determine the nature of the relationship between treatment conditions and the degree of fluorescence within the sub-populations of the cell sample.
Figure 5: Averaged laser scattering data for leukemic samples incubated for 30 min. at $2 \times 10^6$ cells/mL with 15 uL of ALDEFLUOR© reagent. Purple, green, and blue dots indicate correspondence to populations 1, 2, and 3 respectively. Black dots indicate those located outside of border of the selected regions.

Further Measures & Conclusions

When it began, this project had several research aims. First, it aimed to establish ALDEFLUOR® as an alternative method for exclusively identifying leukemia in the hemolymph of soft-shell clams. A second goal was to identify the exact protocol conditions for which an ideal separation of control and test sample fluorescence would take place. The third and final goal of the project was to compare and contrast ALDEFLUOR® with the traditional visualization method in terms of timing, cost, and overall effectiveness.

Unfortunately, it does appear that the first goal of this project was ever truly realized. The generalized trends as described in the discussion section are slim correlations across a wide range of variables. The biochemical justifications for many of the observed phenomena are shaky, and as such there are no clear descriptions of the behavior of the system. Without being told the leukemic/non-leukemic status of the clam ahead of time, it would be impossible to determine that status from examining the set of scatterplots and histogram data alone.

Due to the finicky nature of the leftward shift phenomenon, and due to the surprising results of the change in reagent concentration, it is clear that many more tests need to be done to conclusively determine the ideal conditions required to produce a maximal separation of experiments and controls. However, we can reach a few tentative conclusions in this regard. For leukemic samples, the conditions which generated the largest rightward shift and smallest degree of histogram overlap for both populations was incubation at $2 \times 10^6$ cells/mL for 45 minutes. For non-leukemic samples, the conditions which produced these results were incubation at $1 \times 10^5$ cells/mL for 30 minutes. It should be noted that these conclusions are tentative, as they have yet to be tested for changes in reagent concentration.
As for the third goal, it is clear that the traditional microscopy visualization method is the better of the two at this point in time. In terms of finances, the cost for the traditional method is almost nonexistent, as the procedure requires only a 96-well plate and functional compound light microscope (in addition to the supplies required for hemolymph extraction, but these are needed for both methods, and so are excluded from the direct analysis). The financial situation for the ALDEFLUOR® system seems exorbitant by comparison. The kit itself costs over $500, and only contains enough material for 40 tests. When one factors in the costs for the other materials and machines needed to perform the full procedure, the expenses only continue to accumulate. The ALDEFLUOR® system is also a much slower process than the traditional visualization method. On the whole, the experimenter will spend 15-25 minutes on average examining the 96-well plate with microscopy, which in itself is less than the average amount of time needed for incubation alone; in total, the experimenter can spend up to 6 hours running the entire ALDEFLUOR® protocol from start to finish.

These disadvantages may have been mitigated though, had the ALDEFLUOR® assay worked more effectively than the traditional method. Alas, this does not appear to be the case at this point in time. It is possible that further research may uncover a stronger correlation between the stage of leukemia and ALDH activity than has been covered here thus far. In addition to changing reactant concentration, a few other variables in protocol remain open for additional testing, including the use of efflux inhibitors to prevent escape of the converted reagent from the cells into solution, as well as the temperature of incubation, and the length of time between resuspension and flow cytometry analysis.

Furthermore, as the question of the multiple sub-populations has yet to be fully resolved, this also serves as an area for further research. Until these questions are resolved, however, it is
the strong recommendation of the experimenter is to continue the use of the traditional microscopic method for identifying leukemia in soft-shell clams.

**Acknowledgements**

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References


