Single Animal Dissociation for Whole-animal, Single-cell Molecular Analysis

Angelica Helton[1], Guillaume Aubry[2], Gongchen Sun[2], and Dr. Hang Lu[2]

[1]Department of Biomedical and Chemical Engineering, Florida Institute of Technology
[2]Department of Chemical and Biomolecular Engineering, Georgia Institute of Technology

Abstract

It is readily observable that individuals in a population often respond differently to the same stimulus. This is particularly relevant in therapeutics where patients react differently to a same treatment. To better understand this heterogeneity requires studying gene expression of individuals and the use of small model organisms such as *C. elegans* that share a large genetic homology with humans. Current methods using *C. elegans* nematodes have focused on studying the transcriptome at the population level; however, none are adapted to assess the transcriptome of individuals. Therefore, new tools are needed to transition from population to single-animal level analysis; moreover, new methods are required to dissociate single individuals into single cells. To do so, numerous challenges need to be addressed, including the elimination of centrifugation, reduction of reagents, isolation of tissue types, preservation of high dissociation efficiency, and prevention of cell loss. This research addresses these challenges by developing an adapted dissociation protocol to handle single animals. In addition, by using transgenic strains, we show that different tissue types can be dissociated at the single animal level. Finally, quantification highlights the possibility for improved dissociation efficiency through optimization of incubation times. Our method paves the way for performing transcriptomics of single animals at single-cell resolution.

Introduction

Single-cell transcriptomics studies have known a large success to reveal gene expression and characterize different cell types. One attractive goal remains in linking gene expression to the phenotype of single individuals in order to understand heterogeneity underlying these phenotypes. Such research would benefit the field of precision medicine. [1] In situations where patients react differently to the same treatment, understanding the molecular basis may help develop improved personalized therapeutic treatments. *Caenorhabditis elegans*, a small nematode that shares large genetic homology with humans, is an interesting model organism for this research due to its low cell count, soft-tissue composition, and ease for transgenic manipulation.

While there are multiple methods to study the transcriptome, single cell characterization through RNA sequencing is increasingly popular as it allows for sequencing of the full mRNA strand. [2] The process of single cell characterization includes the dissociation of cells, isolation of mRNA, library preparation and sequencing. Current transcriptomic techniques using *C. elegans* are performed at the population level, containing ten to fifty thousand animals. [3,4] Therefore, sequenced RNA can be linked only to population-level not individual behavior. Furthermore, the use of population dissociation relies on the multitude of cells to provide RNA. In order to determine the transcriptome of single animals with single-cell characterization, new tools are needed. Challenges must be addressed, such as handling single animals, reducing reagents, isolating tissue types, maintaining high dissociation efficiency, and avoiding cell loss. In this paper we adapted a protocol to handle single animal dissociation and demonstrate dissociation of muscle tissue and neurons specifically.

Methods

***C. elegans* Strains and Culture.** Worms were cultured on nematode growth medium (NGM) plates seeded with OP50 *Escherichia coli* bacteria using standard methods. [3] The wild-type strain was N2. The transgenic strains were OH15500: otIs669;otIs672, AQ2954: ljIS131[pmyo-3:GCaMP3-SL2-tagRFP-T]IV, and RW10006: unc-119 (ed3) ruIs32 III; zuIs178 V. Populations were age synchronized through bleaching.

Adult Cell Dissociation. The developed protocol is derived from [4]. All reagents were reduced to 10% of normal quantity. The dissociation was performed in microwells created using PDMS-glass technology. A 5 mm biopsy punch was used to create wells at equal distances in a PDMS slab which was then plasma bonded to a glass slide. Young adult worms were picked into wells containing M9 and washed 4x with M9 to remove bacteria. The animal was washed with 50 µL Lysis buffer (200 mM DTT, 0.25% SDS, 20 mM HEPES pH 8.0, 3% sucrose) and suspended in 75 µL Lysis buffer. Individuals were incubated in at room temperature for 2 minutes without agitation. Each individual was washed 5x with M9 and suspended in 10 mg/mL pronase. Worms were incubated at room temperature for 3-9 minutes with mechanical agitation by pipette 100-120x per minute. To increase throughput, a multi-channel pipette was used on 8 animals at once. The reaction was stopped using ice-cold 2% FBS in PBS.

Imaging and Analysis. Images were taken immediately after dissociation at varying magnifications on an epifluorescence microscope. Micromanager and LLE 7 channel Spectrum GUI were used to control imaging conditions. A cyan fluorescence source was used for the AQ2954 and RW10006 strains, and a green fluorescence source was used for the OH15500 strain. Dissociation efficiency was quantified by counting fluorescent cells at 10x magnification.

Results and Discussion

First, we established the method for dissociating single animals by comparing the results of our PDMS well device with the bulk assay performed in centrifuge tube. Three dissociations were performed on wild-type animals to determine a baseline. Figure 1 shows a population dissociation, a 10-worm dissociation with decreased pronase time, and a single-animal dissociation from left to right. It is notable that Figures 1a and 1c are similar in appearance. This qualitatively shows that we reach similar dissociation results at the single level. Alternatively, Figure 1b shows residual tissue from insufficient dissociation. The clumping seen in this image is attributed to the significant decrease in pronase time for this trial. 2 minutes Lysis Buffer incubation time was deemed appropriate for reduced animal quantities by observation of worm movement.

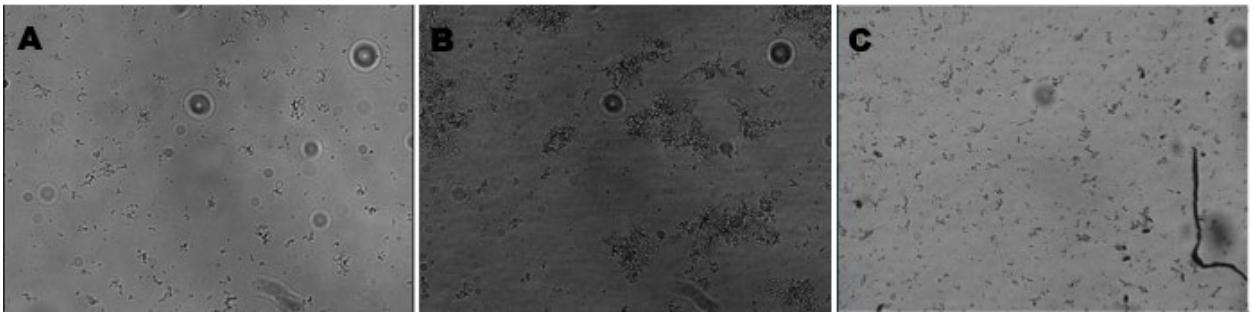

Figure 1: **Images from the dissociations of wild-type animals. A.** Population dissociation with 10 minutes of pronase treatment. This result is used as baseline for comparison with dissociations in different conditions. **B**. Dissociation of 10 animals with 3.5 minutes of pronase treatment which shows cell clumping/ undissociated tissue as

well as fully dissociated cells. **C.** Single animal dissociation with 10 minutes of pronase treatment showing similar results to population dissociation. All images are taken at 20x magnification in brightfield modality.

The histone-tagged fluorescent strain, RW10006, was used for quantification of dissociation efficiency, *i.e.,* the percent of cells recovered after dissociation. Calculating this percentage is possible because hermaphroditic, young-adult *C. elegans* have a set number of 959 somatic cells. Results shown in Table 1 show a maximum efficiency of 53.2% and standard deviation of 1.9%. Reasons for non-optimal efficiency include cell lysis during dissociation, cell loss during transfer, and undercounting. These results can be improved through optimization of lysis buffer and pronase incubation time.

Table 1: Dissociation efficiency of pan-cell strain through fluorescence counting.

Trial	Pronase Time	Slide cell count	Estimated total	Dissociation Efficiency
1	8 min	46	230	24.0%
2	6 min	95	475	49.5%
3	6 min	102	510	53.2%
4	6 min	97	485	50.6%

Studying neuromuscular disorders of *C. elegans* provides insight on Parkinson's, amyotrophic lateral sclerosis, Huntington's, and multiple sclerosis. [6] Due to these applications, we focused on recovery of muscle and neuronal cells. We used transgenic strains with fluorescent muscle or neuron cells and compared the outcome of a population dissociation with single-animal dissociation. As seen in Figure 2, some neuron and muscle cells were successfully dissociated as indicated by the presence of fluorescent spots in the images. The decrease from three to one fluorescent spots in images A and B respectively results from reduced animal and reagent quantity so it is expected. This proof-of-concept of dissociating neurons and muscle cells is highly interesting as it opens opportunities for studying neuromuscular diseases.

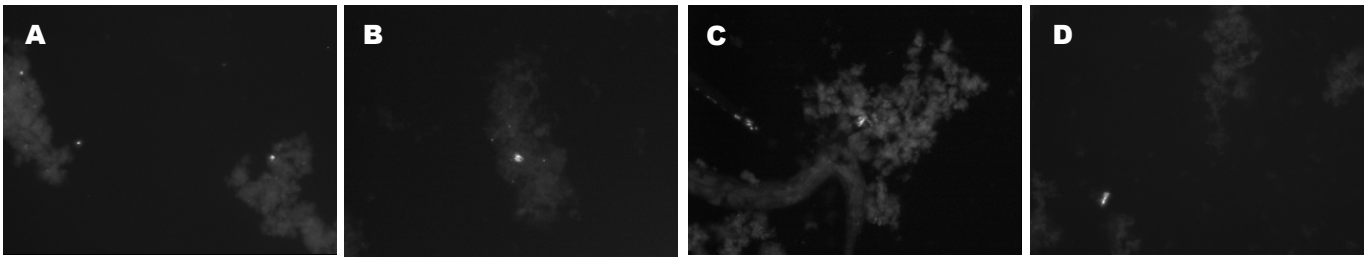

Figure 2: Images of transgenic strains showing tissue type can be identified at the single-animal level. **A**. Population dissociation of AQ2954 strain showing three fluorescent spots for muscle cells. **B**. Single animal dissociation of AQ2954 showing singular fluorescent spot of the same brightness as population dissociation. **C**. Population dissociation of OH15500 showing partially dissociated worm and fluorescent spot in tissue cloud. **D**. Single animal dissociation of OH15500 which shows singular fluorescent spot and successful isolation of neurons.

Conclusion and Future Work

This work shows that single-animal dissociations can be performed despite challenges such as manipulating single animals and low amounts of reagents and preserving specific tissue types. We have successfully handled single animals by creating microwells through PDMS-glass technology.

In addition, we dissociated single animals and showed up to 53% single cell recovery. Finally, we demonstrated, through transgenic strains, that single cells dissociated from single animals originated from tissues such as muscles and neurons, which are of particular interest for behavior and neuroscience studies. This method serves as the basis for tissue preparation before RNA sequencing and will be instrumental to studying the molecular basis of heterogeneity in macroscopic phenotypes.

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Contact

hang.lu@gatech.edu

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