Functional Analysis of Protonephridia Genes Identified by Drop-seq

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Introduction

Planarians possess a primitive excretory system called protonephridia, which consists of an organized network of branched tubes that function in waste filtration and water regulation. On a cellular and molecular level, the planarian excretory system shows considerable homology to the human excretory system: the kidney. Knowing what genes are necessary for a functional excretory system, how the genes interact, and mechanisms that alter normal gene function leading to disease is critical for the development of treatments for excretory system diseases. Furthermore, understanding flatworm-specific genes, many of which are parasitic to humans, may provide useful drug targets for treating parasitic worm infections.

A recent, significant advancement has been the development of single-cell transcriptomic technologies that allow for the ability to examine gene expression profiles of individual cells (Figure 1). Planarians were the first organism to have these technologies applied to determine the expression profile of all of the animal's cells (Fincher, 2018). However, only a few of the 3,000 protonephridia genes identified and available via the public database (https://digiworm.wi.mit.edu/) were validated by Fincher et al. To further explore the genes required for excretory system function, we have chosen the 48 most highly expressed protonephridia genes from the Fincher 2018 paper to analyze via bioinformatics, RNA mediated interference, and in situ hybridization.

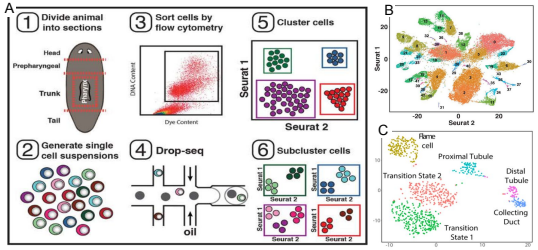
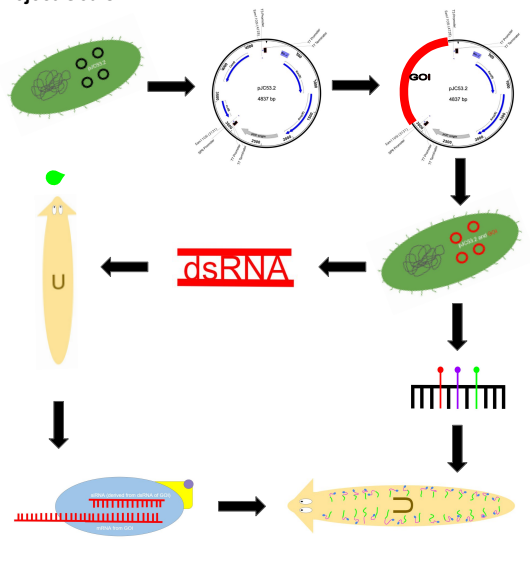


Figure 1: Single-cell transcriptomic data generated by Fincher et al. (2018) using Drop-seq. (A) Schematic illustrating the Drop-seq method used to isolate, cluster, and assign gene expression profiles to single cells. (B) 44 planarian cell type clusters representing each cell in the organism. (C) Protonephridia cell clusters.

Project Goals



Contig	Name	Human / Flatworm BLAST	E value
>dd_smedV4_951_0_1	418	Potential peptide hormone	3e-8
>dd_smedV4_2210_0_1	419	ATP1B1	2e-55
>dd_smedV4_16743_0_1	420	Small peptide	N/A
>dd_smedV4_17_0_1	421	Thymosin beta	3e-14
>dd_smedV4_11240_0_1	422	ZBTB40	1e-6
>dd_smedV4_2698_0_1	423	TSPAN3	4e-23
>dd_smedV4_6287_0_1	424	Coil domain protein	5e-29
>dd_smedV4_10214_0_1	425	Spectrin repeat protein	4e-132
>dd_smedV4_1571_0_1	426	Novel flatworm gene	7e-129
>dd_smedV4_5028_0_1	427	PIK3C3	2e-144
>dd_smedV4_3820_0_1	428	Coil domain protein	7e-177
>dd_smedV4_2189_0_1	429	HSP20	3e-6
>dd_smedV4_4660_0_1	430	Novel flatworm gene	6e-31
>dd_smedV4_8220_0_1	431	Plectin	4e-7
>dd_smedV4_15904_0_1	432	SYNE1	4e-90
>dd_smedV4_10646_0_1	433	Microtubule-actin crosslink	2e-55
>dd_smedV4_3340_0_1	434	ATP1A3	0
>dd_smedV4_6707_0_1	435	FhCaBP2	7e-17
>dd_smedV4_3732_0_1	436	CAFP45	8e-97
>dd_smedV4_10072_0_1	437	PLEKHG5	1e-56
>dd_smedV4_299_0_1	438	CALML5	2e-52
>dd_smedV4_7716_0_1	439	Secreted protein	1e-60
>dd_smedV4_4389_0_1	440	SLC12 member 5	0
>dd_smedV4_9904_0_1	441	SLC8B1	7e-41
>dd_smedV4_6156_0_1	442	DOCK1	9e-68
>dd_smedV4_13612_0_1	443	OTOP1	2e-6
>dd_smedV4_4925_0_1	444	TPTE2	5e-104
>dd_smedV4_817_1_1	445	PGLYRP 2	9e-47
>dd_smedV4_9276_0_1	446	PDE4D	3e-162
>dd_smedV4_4047_0_1	447	SLC12A4	0
>dd_smedV4_777_0_1	448	Novel flatworm gene	3e-27
>dd_smedV4_780_0_1	449	Transmembrane protein	5e-13
>dd_smedV4_10881_0_1	450	RDH13	1e-32
>dd_smedV4_11520_0_1	451	Fimbrin/PLS1	2e-60
>dd_smedV4_4296_0_1	452	RBPMS	6e-23
>dd_smedV4_5620_0_1	453	TNS1	1e-41
>dd_smedV4_1561_0_1	454	Myelin PLP	3e-24
>dd_smedV4_5024_0_1	455	CLF_105639	6e-48
>dd_smedV4_9557_0_1	456	ATP2B4	3e-88
>dd_smedV4_9764_0_1	457	Small peptide	N/A
>dd_smedV4_16329_0_1	458	Microtubule-actin crosslink	1e-8
>dd_smedV4_3219_0_1	459	CFAP53	1e-26
>dd_smedV4_6921_0_1	460	Novel flatworm protein	6e-158
>dd_smedV4_6499_0_1	461	DAB2IP	2e-99
>dd_smedV4_5494_0_1	462	TCTEX-1	2e-14
>dd_smedV4_770_0_1	463	4 pass transmembrane	2e-65
>dd_smedV4_2676_0_1	464	Sperm axonemal protein	1e-21
>dd_smedV4_1131_0_1	465	Moesin	3e-179

Results

We used bioinformatic tools such as BLAST to determine whether each gene had a human homolog or was flatworm-specific. The same technique was also used to generate primers needed to clone each gene (Table 1). Prior to cloning these genes, we needed to improve the transformation efficiency of our cell lines, and to do this we used the Inoue protocol which increased the efficiency by nearly three orders of magnitude. We also confirmed the integrity of the pJC53.2 plasmid cloning vector's CcdB/CcdA toxin-antitoxin system by plating and used PCR to amplify and confirm known segments on the vector. We have begun to amplify candidate genes from planarian cDNA using polymerase chain reaction. Thus far, we have attained an 80% amplification success rate. These PCR products will be inserted into the cloning vector and transformed into our newly prepared DH5a ultra-competent cells to obtain clones of each gene of interest (Figure 2).

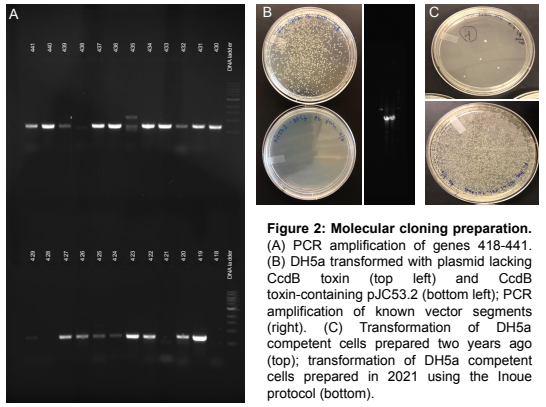


Figure 2: Molecular cloning preparation. (A) PCR amplification of genes 418-441. (B) DH5a transformed with plasmid lacking CcdB toxin (top left) and CcdB toxin-containing pJC53.2 (bottom left); PCR amplification of known vector segments (right). (C) Transformation of DH5a competent cells prepared two years ago (top); transformation of DH5a competent cells prepared in 2021 using the Inoue protocol (bottom).

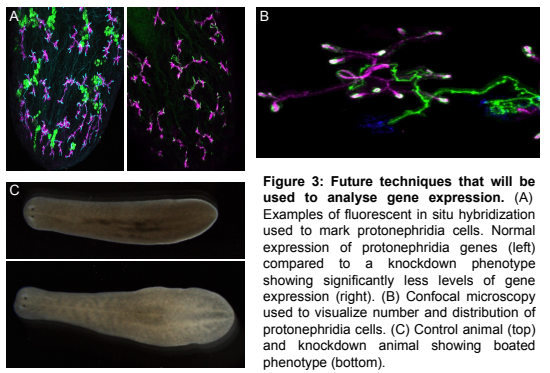


Figure 3: Future techniques that will be used to analyse gene expression. (A) Examples of fluorescent in situ hybridization used to mark protonephridia cells. Normal expression of protonephridia genes (left) compared to a knockdown phenotype showing significantly less levels of gene expression (right). (B) Confocal microscopy used to visualize number and distribution of protonephridia cells. (C) Control animal (top) and knockdown animal showing boated phenotype (bottom).

Future Directions

After cloning all 48 genes, we will generate riboprobes for in situ hybridization which will be used to visualize each gene's expression. This will allow us to validate whether each gene is expressed in protonephridia and map its expression to specific protonephridia cell types. We will also generate gene specific dsRNA for an RNAi screen, which will allow us to identify animals that look swollen, a symptom that commonly occurs when animals are incapable of excreting excess fluid from their tissues. Several staining methods and confocal microscopy will also be used to examine whether gene knockdown animals have normal number and distribution of protonephridia cells (Figure 3). After completing the initial screen of the 48 genes identified by Fincher et al. (2018), we will identify candidate genes showing interesting phenotypes to perform more refined analyses. This includes performing knockdowns with genes of interacting pathways and examining ciliary motility and structure, which is important for proper protonephridia function.

References

Fincher, Christopher T et al. (2018). Cell type transcriptome atlas for the planarian Schmidtea mediterranea. Science, 10.1126/science.aaq1736.