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Comprehensive single cell transcriptional profiling of a multicellular organism

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Abstract

To resolve cellular heterogeneity, we developed a combinatorial indexing strategy to profile the transcriptomes of single cells or nuclei (sci-RNA-seq: Single cell Combinatorial Indexing RNA sequencing). We applied sci-RNA-seq to profile nearly 50,000 cells from the nematode *Caenorhabditis elegans* at the L2 stage, which is over 50-fold “shotgun cellular coverage” of its somatic cell composition. From these data, we define consensus expression profiles for 27 cell types, and recover rare neuronal cell types corresponding to as few as one or two cells in the L2 worm. We integrate these profiles with whole animal ChIP sequencing data to deconvolve the cell type specific effects of transcription factors. These data generated by sci-RNA-seq constitute a powerful resource for nematode biology, and foreshadow similar atlases for other organisms.

Individual cells are the natural unit of form and function in biological systems. However, conventional methods for profiling the molecular content of biological samples mask cellular heterogeneity, likely present even in ostensibly homogenous tissues (1). Recently, profiling the transcriptome of individual cells has emerged as a powerful strategy for resolving such heterogeneity. The expression levels of mRNA species are linked to cellular

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function, and therefore can be used to classify cell types (2–10) and to order cell states (11). Although methods for single cell RNA-seq have proliferated, they rely on the isolation of individual cells within physical compartments (12–20). Consequently, preparing single cell RNA-seq libraries with these methods can be expensive, the cost scaling linearly with the numbers of cells processed (21, 22).

We recently developed combinatorial indexing, a method using split-pool barcoding of nucleic acids to uniquely label a large number of single molecules or single cells. Single *molecule* combinatorial indexing can be used for haplotype-resolved genome sequencing and *de novo* genome assembly (23, 24), while *single cell* combinatorial indexing (“sci”) can be used to profile chromatin accessibility (sci-ATAC-seq) (25), genome sequence (sci-DNA-seq) (26), genome-wide chromosome conformation (sci-Hi-C) (27), and DNA methylation (sci-MET) (28) in large numbers of single cells.

Here we developed a combinatorial indexing method to uniquely label the transcriptomes of large numbers of single cells or nuclei (sci-RNA-seq). We then applied sci-RNA-seq to deeply profile single cell transcriptomes in the nematode *C. elegans* at the L2 stage. *C. elegans* is the only multicellular organism for which all cells and cell types are defined, as is its entire developmental lineage (29, 30). However, despite its modest cell count (*e.g.* 762 somatic cells per L2 larva), our knowledge of the molecular state of each cell and cell type remains fragmentary. We therefore saw an opportunity to generate a powerful resource for nematode biologists as well as for the single cell genomics community.

Overview of sci-RNA-seq

In its current form, sci-RNA-seq relies on the following steps (Fig. 1A): 1) Cells are fixed and permeabilized with methanol (alternatively, cells are lysed and nuclei recovered), and then split across 96- or 384-well plates. 2) A first molecular index is introduced to the mRNA of cells within each well with *in situ* reverse transcription (RT) incorporating a barcode-bearing, well-specific polyT primer containing unique molecular identifiers (UMI). 3) All cells are pooled and redistributed by fluorescence activated cell sorting (FACS) to 96- or 384-well plates in limiting numbers (*e.g.* 10–100 per well). Cells are gated on the basis of DAPI (4',6-diamidino-2-phenylindole) staining to discriminate single cells from doublets during sorting. 4) Second strand synthesis, transposition with Tn5 transposase, lysis, and PCR amplification are performed. The PCR primers target the barcoded polyT primer on one end, and the Tn5 adaptor insertion on the other end, such that resulting PCR amplicons preferentially capture the 3' ends of transcripts. These primers introduce a second barcode, specific to each well of the PCR plate. 5) Amplicons are pooled and subjected to massively parallel sequencing, resulting in 3'-tag digital gene expression profiles, with each read associated with two barcodes corresponding to the first and second rounds of cellular indexing (Fig. 1B). In a variant of the method described below, we introduce a third round of cellular indexing during Tn5 transposition of double-stranded cDNA.

The majority of cells pass through a unique combination of wells, resulting in a unique combination of barcodes for each cell that tags its transcripts. The rate of two or more cells receiving the same combination of barcodes can be tuned by adjusting how many cells are

distributed to the second set of wells (25). Increasing the number of barcodes used during each round of indexing leads to boosts the number of cells that can be profiled while reducing the effective cost per cell (fig. S1). Additional levels of indexing can potentially offer even greater complexity and lower costs. Multiple samples (*e.g.* different cell populations, tissues, individuals, time-points, perturbations, replicates, etc.) can be concurrently processed within one experiment, using different subsets of wells for each sample during the first round of indexing.

Scalability of sci-RNA-seq

We tested 262 sci-RNA-seq conditions with mammalian cells, optimizing the protocol and reaction conditions. We demonstrate scalability with 384×384 well sci-RNA-seq. During the first round of indexing, half of 384 wells contained pure populations of either human (HEK293T or HeLa S3) or mouse (NIH/3T3) cells, and the other half mixed human and mouse cells (Table S1). After barcoded RT, cells were pooled and then sorted to a new 384 well plate for the second round of barcoding and deep sequencing of pooled PCR amplicons. We recovered 15,997 single cell transcriptomes and readily assigned cells as human or mouse (Fig. 1C).

Optimization of sci-RNA-seq and application to nuclei

We performed optimized 96×96 well sci-RNA-seq on five cell populations, each present in distinct subsets of wells during the first round of barcoding (Table S1): HEK293T cells (8 wells); HeLa S3 cells (8 wells); an intraspecies mixture of HEK293T and HeLa S3 cells (32 wells); and interspecies mixtures of HEK293T and NIH/3T3 cells (24 wells) or nuclei (24 wells). We deeply sequenced the resulting library (~250,000 reads per cell; ~210,000 reads per nucleus; ~88% duplication rate), profiling 744 single cell and 175 single nucleus transcriptomes.

Transcriptomes in the 24 wells containing an interspecies mixture of human and mouse cells overwhelmingly mapped to the genome of one species or the other (289 of 294 cells), with only 5 ‘collisions’ (where collisions likely represent coincidental passage through the same wells by two or more cells) (Fig. 1D). Excluding collisions, we observed an average of 24,454 UMIs (5,604 genes) per human cell and 17,665 UMIs (4,065 genes) per mouse cell, with 1.9% and 3.3% of reads per cell mapping to the incorrect species.

Transcriptomes originating in the 24 wells containing an interspecies mixture of human and mouse nuclei also overwhelmingly mapped to the genome of one species or the other (172 of 175 nuclei), with only 3 collisions (fig. S2A). Excluding collisions, we observed an average of 32,951 UMIs (5,737 genes) per human nucleus and 20,123 UMIs (4,107 genes) per mouse nucleus (fig. S2B–C), with 2.2% and 1.9% of reads per cell mapping to the incorrect species. The greater UMI counts in nuclei are potentially due to the higher amounts of mRNA in cells resulting in a reduced RT efficiency per molecule. Consistent with this, optimizing the number of cells per RT reaction increased UMI counts per cell (31).

Estimates of gene expression from the aggregated transcriptomes of nuclei versus cells were well correlated (Pearson: 0.96 for HEK293T, 0.97 for NIH/3T3; Fig. 1E, fig. S2D). From

cells, 81% of reads mapped to the expected strand of genic regions (47% exonic, 34% intronic), and 19% to intergenic regions or the unexpected strand of genic regions. From nuclei, 84% of reads mapped to the expected strand of genic regions (35% exonic, 49% intronic) and 16% to intergenic regions or the unexpected strand of genic regions, similar to previous studies (32). Whereas exonic reads show an expected enrichment at the 3' ends of gene bodies, intronic reads do not, and may be the result of poly(dT) priming from poly(dA) tracts in heterogeneous nuclear RNA (fig. S3).

Transcriptomes originating in the 48 wells containing pure or an intraspecies mixture of HEK293T and HeLa S3 cells were readily separated into two clusters by t-stochastic neighbor embedding (t-SNE) (Figs. 1F and S4). Estimates of gene expression from the aggregated transcriptomes of all identified HEK293T cells versus a related bulk RNA-seq workflow (Tn5-RNA-seq (33)) without methanol fixation were well correlated (Pearson: 0.94, Fig. 1G).

Robustness of sci-RNA-seq

After optimizing the number of cells per RT reaction, we fixed a mixture of HEK293T and NIH/3T3 cells, and performed 16×84 well sci-RNA-seq (Table S1) (31). We recovered 185 human cells and 109 mouse cells with 22 collisions (Fig. 2A). At $\sim 240,000$ reads per cell (73% duplication rate), we observed an average of 49,043 UMIs (7,563 genes) per human cell and 36,737 UMIs (6,263 genes) per mouse cell (Fig. 2B, fig. S5A), with 0.9% and 1.2% of reads per cell mapping to the incorrect species. Although this and the previous experiment were performed two months apart on independently grown and fixed cells, the aggregated transcriptomes were well correlated (Pearson: 0.98 for HEK293T, 0.98 for NIH/3T3; Figs. 2C and S5B).

We stored a portion of the methanol-fixed mixture of HEK293T and NIH/3T3 cells at -80°C for 4 days and repeated sci-RNA-seq (Table S1). At $\sim 200,000$ reads per cell (73% duplication rate), we observed an average of 30,024 UMIs (5,965 genes) per human cell and 21,393 UMIs (4,503 genes) per mouse cell, with comparable purity (fig. S5C). The aggregated transcriptomes of the fixed-fresh vs. fixed-frozen cells were well correlated (Pearson: 0.99 for HEK293T cells, 0.98 for NIH/3T3 cells; Figs. 2D and S5D).

sci-RNA-seq with three levels of indexing

Two-level combinatorial indexing enables routine profiling of $\sim 10^4$ single cells per experiment. We tested an additional level of indexing during Tn5 transposition of double-stranded cDNA (25). We performed $16 \times 6 \times 16$ well sci-RNA-seq on mixed HEK293T and NIH/3T3 cells after methanol fixation. After RT with 16 barcodes and second strand synthesis, cells were pooled and distributed to 6 wells for tagmentation with indexed Tn5 (6 barcodes), then pooled again and sorted to 16 wells for PCR with indexed primers. At $\sim 20,000$ reads per cell (51% duplication rate), we recovered 119 human and 62 mouse cells with 5 collisions (fig. S6A). The aggregated transcriptomes of three-level vs. two-level sci-RNA-seq were well correlated (Pearson: 0.96 for HEK293T, 0.94 for NIH/3T3; fig. S6B–C). Downsampling to 15,000 reads per cell, three-level indexing recovered fewer UMIs per cell

than two-level indexing (3-level: on average, 6,033 for HEK293T, 3,640 for NIH/3T3; 2-level: 9,942 for HEK293T, 8,611 for NIH/3T3; fig. S6D–G), possibly due to lower efficiency of indexed vs. unindexed Tn5. This limitation notwithstanding, three-level combinatorial indexing has the potential to enable routine profiling of $>10^6$ single cells per experiment (fig. S6H; (31)).

Single cell RNA profiling of *C. elegans*

We next applied sci-RNA-seq to *C. elegans*. Of note, the cells in *C. elegans* larvae are much smaller, more variably sized, and have lower mRNA content than the mammalian cell lines on which we optimized the protocol. We pooled ~150,000 larvae synchronized at the L2 stage and dissociated them into single-cell suspensions. We then performed *in situ* RT across six 96-well plates (576 first-round barcodes), each well containing ~1,000 *C. elegans* cells and also ~1,000 human cells (HEK293T) as internal controls. After pooling all cells, we sorted the mixture of *C. elegans* and HEK293T cells to 10 new 96-well plates for PCR barcoding (960 second-round barcodes), gating on DNA content to distinguish between *C. elegans* and HEK293T cells. This sorting resulted in 96% of wells harboring only *C. elegans* cells (140 each), and 4% of wells harboring a mix of *C. elegans* and HEK293T cells (140 *C. elegans* and 10 HEK293T each).

This experiment yielded 42,035 *C. elegans* single-cell transcriptomes (UMI counts per cell for protein-coding genes = 100). 94% of reads mapped to the expected strand of genic regions (92% exonic, 2% intronic). At a sequencing depth of ~20,000 reads per cell and a duplication rate of 80%, we identified a median of 575 UMIs mapping to protein-coding genes per cell (mean 1,121 UMIs and 431 genes per cell) (fig. S7A). Importantly, control wells containing both *C. elegans* and HEK293T cells demonstrated clear separation between species (fig. S7B), with 3.1% and 0.2% of reads per cell mapping to the incorrect species, respectively.

Identifying cell types

Semi-supervised clustering analysis segregated the cells into 29 distinct groups, the largest containing 13,205 (31.4%) and the smallest only 131 (0.3%) cells (Fig. 3A). Somatic cell types comprised 37,734 cells. We identified genes that were expressed specifically in a single cluster, and by comparing those genes to expression patterns reported in the literature, assigned the clusters to cell types (figs. S15–S23). Twenty-six cell types were represented in the 29 clusters: 19 represented exactly one literature-defined cell type, 7 contained multiple distinct cell types, 2 contained cells of a specific cell type but had abnormally low UMI counts, and 1 could not be readily assigned. Neurons, which were present in 7 clusters in the global analysis, were independently reclustered, initially revealing 10 major neuronal subtypes.

Intestine cells were not represented in any cluster. Intestine cells comprise 2.5% of the somatic cells but are polyploid in *C. elegans* larvae (34) and also autofluorescent in the DAPI channel used to measure DNA content (35). We speculated they may have been excluded by how we gated on DNA content. We therefore performed a second 384×144

well *C. elegans* experiment, collecting all cells including polyploid cells on the basis of DAPI fluorescence (96 wells), or gating to enrich for polyploid cells (48 wells). Intestine cells were present (as compared with their absence in the previous experiment) and 2-fold enriched in wells gated for polyploidy. This experiment yielded 7,325 cells (UMI counts per cell for protein-coding genes = 200), of which 6,335 were somatic and 511 intestine cells (fig. S8A).

Gene expression patterns in hypodermal cells suggested that the worm cells from the second *C. elegans* experiment were more tightly synchronized, overlapping but not identical in developmental timing to the first experiment (fig. S8B–F). *C. elegans* larvae feature pervasive oscillations in gene expression within each larval stage (36), making it difficult to distinguish biological variation from batch effects. However, the aggregated transcriptomes of human HEK293T cells from these same experiments were well correlated (Pearson: 0.97) and not readily separated by tSNE (fig. S9). This suggests that the variation observed is primarily due to differences in the developmental timing or preparation of the *C. elegans* larvae and cells, rather than technical variation in the sci-RNA-seq protocol. Regardless of its source, to minimize confounding by this variation, we only included the intestine cells from the second *C. elegans* experiment in subsequent analyses, with all other cell types being represented by the first experiment only.

The global and neuron-specific clustering analyses from the first *C. elegans* experiment, supplemented with intestine cells from the second experiment, allowed us to construct aggregate expression profiles for 27 cell types (Tables S2–S4; a 28th cell type, dopaminergic neurons, is excluded due to small cell numbers). These profiles are available online via GExplore (http://genome.sfu.ca/gexplore/gexplore_search_tissues.html; fig. S14). Comparing the observed proportions of each cell type to their known frequencies in L2 larvae showed that sci-RNA-seq captured many cell types at or near expected frequencies (Fig. 3B; 15/28 types had abundance > 50%, and 27/28 had abundance > 20%, of expectation).

Transcriptional programs can be readily distinguished within single cell transcriptome datasets at shallow sequencing depths (37). Thus, despite being able to distinguish many distinct cell types in the worm, our molecular definition for each would be incomplete. However, we observed that half of all *C. elegans* protein-coding genes were expressed in at least 100 cells in the full dataset, and 66% of protein-coding genes in at least 20 cells. This compares favorably with the estimates of expressed genes at the L2 stage from whole animal RNA-seq (69%) (38). The “whole worm” expression profile derived by aggregating all sci-RNA-seq reads correlated well with whole animal bulk RNA-seq (38) for L2 *C. elegans* (Fig. 3C; Spearman: 0.796 with cells from the first experiment only, 0.824 including intestine cells from the second experiment). Furthermore, 3,925 genes were enriched in a single tissue (differential expression at least five-fold greater than the 2nd-highest expressing tissue; Fig. 3D, Table S6), and 1,939 genes were enriched for expression in a single cell type (Fig. 3E, Table S7). Thus, despite the fact that sci-RNA-seq captures a minority of transcripts in each cell, our ‘oversampling’ of the cellular composition of the organism enables us to construct representative expression profiles for individual cell types (Fig. 3F).

Neuronal cell types

Because the transcripts of tissue or cell type clusters suggested subclasses within groups (Fig. 4A), we examined expression within several tissues in more detail. We confirmed and extended findings that anterior and posterior body wall muscle have distinct expression patterns (fig. S10A–B, Table S9, (39)), and also observed distinct expression patterns for posterior vs. other intestine cells (fig. S10C–D, Table S10) and amphid vs. phasmid sheath cells (fig. S10E–F, Table S11). But gene expression patterns were particularly diverse in neuronal cell types.

By morphological criteria, the 302 neurons of worm are classified into 118 distinct types (40) and from the database of reporter transgene expression patterns, most of these are postulated to have unique molecular signatures (41). Our initial re-clustering of neuronal cells divided them into 10 broad classes (Fig. 4A). Most classes of neurons were represented by several small but highly distinct clusters in the t-SNE plot. Further analysis of cluster-specific gene expression showed that many clusters corresponded to highly specific subsets of neurons in the L2 worm (Fig. 4B, Table S7). Three clusters corresponded to sets of four neurons in an individual worm, 8 clusters corresponded to a single pair of neurons (AFD, ASG, ASK, AWA, BAG, CAN, RIA, and RIC), and 3 clusters corresponded to exactly one neuron (ASEL, ASER, and DVA). Hierarchical clustering analysis showed that of the most of 917 genes highly enriched in neurons, compared to other tissues, were expressed in only a minority of neuronal clusters (Fig. 4C). 73% of neuron-enriched genes had no more than 10 neuron clusters (out of 40 total) in which they were expressed at 10% of the level of the highest-expressed cluster. 155 genes were highly enriched in a single neuron cluster relative to all others (Fig. 4D, Table S8).

Expression of marker genes, such as *gcy-3* and *gcy-6*, were key in identifying two neuronal clusters as left ASE (ASEL) and right ASE (ASER) gustatory neurons, respectively (Fig. 4E). These neurons have asymmetry in gene expression (42), and we observe 44 genes to be differentially expressed (Fig. 4F, Table S12, fold difference > 3, FDR < 5%). mRNA from these neurons has previously been profiled with co-immunoprecipitation of RNA and a transgenic poly(A)-binding protein expressed specifically in ASEL or ASER, followed by microarray analysis (43). The differentially expressed genes we observe are consistent with this study (fig. S11), highlighting the ability of sci-RNA-seq to facilitate the analysis of cell types as rare as a single cell per individual.

Two neuronal clusters correspond to sister cells, the AWA and ASG neurons, (Fig. 4G), which arise from the same parental cell in the last round of *C. elegans* embryonic cell divisions. Their differentiation has previously been used as a model for the study of the regulation of cell fate decisions (44). In our data, 136 genes were differentially expressed between these two cell types (Fig. 4H, Table S13, fold difference > 3, FDR < 5%). The divergent transcriptomes of the AWA and ASG neurons, along with the left and right ASE neurons, highlight the potential of cells that are extremely closely related in morphology and developmental lineage to feature distinct programs of gene regulation.

Integration with transcription factor binding sites

We hypothesized that correlating transcription factor (TF) binding patterns—profiled in ChIP-seq experiments from the modENCODE (45) and modERN (46) consortia—with cell type gene expression profiles could give insights into the regulatory programs underlying the gene expression profiles. For each of 27 cell types, we constructed regularized regression models to predict each gene's expression as a function of the TF ChIP peaks present in its promoter (Fig. 5). We restricted a cell type's model to those TFs that were detectably expressed within it (>10 transcripts per million (TPM)), increasing the proportion of TF-to-cell-type associations that are likely to reflect causal gene regulation. Our regression analysis predicted gene expression by selecting numerous regulators critical for development or proper function-specific cell types, including *hlh-1* and *unc-120* in body wall muscle (47), *pha-4* in pharyngeal cell types (48), *hlh-8* (CeTwist) in sex myoblasts (49), *blmp-1* and *nhr-25* in hypodermis (50, 51), *elt-2* in the intestine (52), and *xnd-1* in the germline (53, 54).

The regression identified several putative novel regulators of cell-type specific expression. For example, *fkf-8*, which is expressed specifically in ciliated sensory neurons (our data and reporter construct from (55)) was predictive of their gene expression program (fig. S12). The uncharacterized TF *F49E8.2* is expressed specifically in the germline and associated with germline gene expression (fig. S12). *F49E8.2* is an ortholog of the human gene “E2F-associated phosphoprotein” (EAPP) (56), and *F49E8.2* ChIP-seq peaks co-localize with germline-specific EFL-1 peaks (ortholog of E2F, data from (57)) more often than could be expected due to chance (fig. S13ab, χ^2 -test, $p = 2.8 \times 10^{-21}$), suggesting that these proteins may physically interact. The hypodermis-associated TFs *blmp-1* and *nhr-25* were also associated with gene expression in socket cells, excretory cells, and rectal cells. *nhr-25* is expressed 4.5-fold higher in socket cells than in seam cells (560 vs. 124 TPM) and 8.7-fold more than in the non-seam hypodermis (560 vs. 64 TPM), suggesting a role in glial development.

Discussion

Our method for single cell RNA-seq combinatorial indexing of cells or nuclei can be applied to profile the transcriptomes of tens-of-thousands of single cells per experiment through a library construction completed by a single individual in two days at a cost of \$0.03-\$0.20 per cell. sci-RNA-seq is compatible with cell fixation, which can minimize perturbations to cell state or RNA integrity before or during processing and facilitates the concurrent processing of multiple samples within a single experiment, potentially reducing batch effects relative to platforms requiring serial processing, an area of concern for the single cell RNA-seq field (58). Given that the second barcode is introduced after flow sorting, it is also possible to associate wells on the PCR plate with FACS-defined subpopulations. sci-RNA-seq is also compatible nuclei, which may be important for tissues for which unbiased cell disaggregation protocols are not well established (possibly most tissues). Lastly, sci-RNA-seq is scalable. We demonstrate up to 576×960 indexing, which enabled the generation of $\sim 4 \times 10^4$ single cell transcriptomes in one experiment. However, processing of more cells with sub-linear cost scaling is possible by using more barcoded RT and PCR primers (e.g. $1,536 \times 1,536$ combinatorial indexing) and/or introducing additional rounds of indexing.

With $384 \times 384 \times 384$ combinatorial indexing, one can hypothetically profile the transcriptomes of over 10 million cells per experiment.

With sci-RNA-seq we generated a catalog of single cell transcriptomes with over 50-fold “shotgun cellular coverage” of the L2 *C. elegans* soma. We detect 18 non-neuronal cell types and a multitude of neuronal cell types, which we grouped into either 10 broad classes or 40 fine-grained clusters from an unsupervised analysis, highlighting the potential of an organism’s gene regulatory programs to be enacted at a fine-grained level. We anticipate these data will be a rich resource for nematode biology – a starting point for an atlas that leverages Sulston’s lineage map to define the molecular state of every cell throughout the life cycle of *C. elegans*. Furthermore, as illustrated by our experience with intestinal cells, the greater knowledge of “ground truth” for *C. elegans* may further the refinement of experimental and computational methods for recovering and distinguishing cell types and states.

sci-RNA-seq expands the repertoire of single cell molecular phenotypes that can be resolved by combinatorial indexing (25–28). Provided that multiple aspects of cellular biology can be concurrently barcoded, combinatorial indexing may also facilitate the scalable generation of ‘joint’ single cell molecular profiles (*e.g.* RNA-seq and ATAC-seq from each of many single cells). We also envision that large-scale, integrated profiling of the molecular states and lineage histories (59) of single cells in other organisms will begin to give shape to “global views” of their developmental biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The raw data have been deposited with the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under accession code GSE98561 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wduvwoqvrynngt&acc=GSE98561>). These data are also made available as gene-by-cell matrices, along with a “vignette” in the form of a Jupyter notebook that shows examples of how to work with the data (http://waterston.gs.washington.edu/sci_RNA_seq_gene_count_data). We thank members of the Shendure, Trapnell and Waterston labs for helpful discussions and feedback, particularly A. Hill, V. Agarwal, M. Gasperinin, L. Starita, Y. Yin and B. Martin; S. Zimmerman and C. Berg for helpful technique suggestions; the modERN consortium for allowing us to use their ChIP-seq data; D. Prunkard, and L. Gitari in the Pathology Flow Cytometry Core Facility for their exceptional assistance in flow sorting; J. Rose, D. Maly, L. VandenBosch, and T. Reh lab for sharing the NIH/3T3 cell line; H. Hutter for adding our tissue-specific expression profiles on gExplore. HeLa S3 cells were used as part of this study. H. Lacks, and the HeLa cell line that was established from her tumor cells in 1951, have made significant contributions to scientific progress and advances in human health. We are grateful to H. Lacks, now deceased, and to her surviving family members for their contributions to biomedical research. This work was funded by grants from the NIH (DP1HG007811 and R01HG006283 to JS; U41HG007355 and R01GM072675 to RHW, DP2 HD088158 to CT) and the Paul G. Allen Family Foundation (to JS), W. M. Keck Foundation (to CT and JS), Dale F. Frey Award for Breakthrough Scientists (to CT) and Alfred P. Sloan Foundation Research Fellowship (to CT) and by the William Gates III Endowed Chair in Biomedical Sciences (RHW). DAC was supported in part by T32HL007828 from the National Heart, Lung, and Blood Institute. JS is an Investigator of the Howard Hughes Medical Institute.

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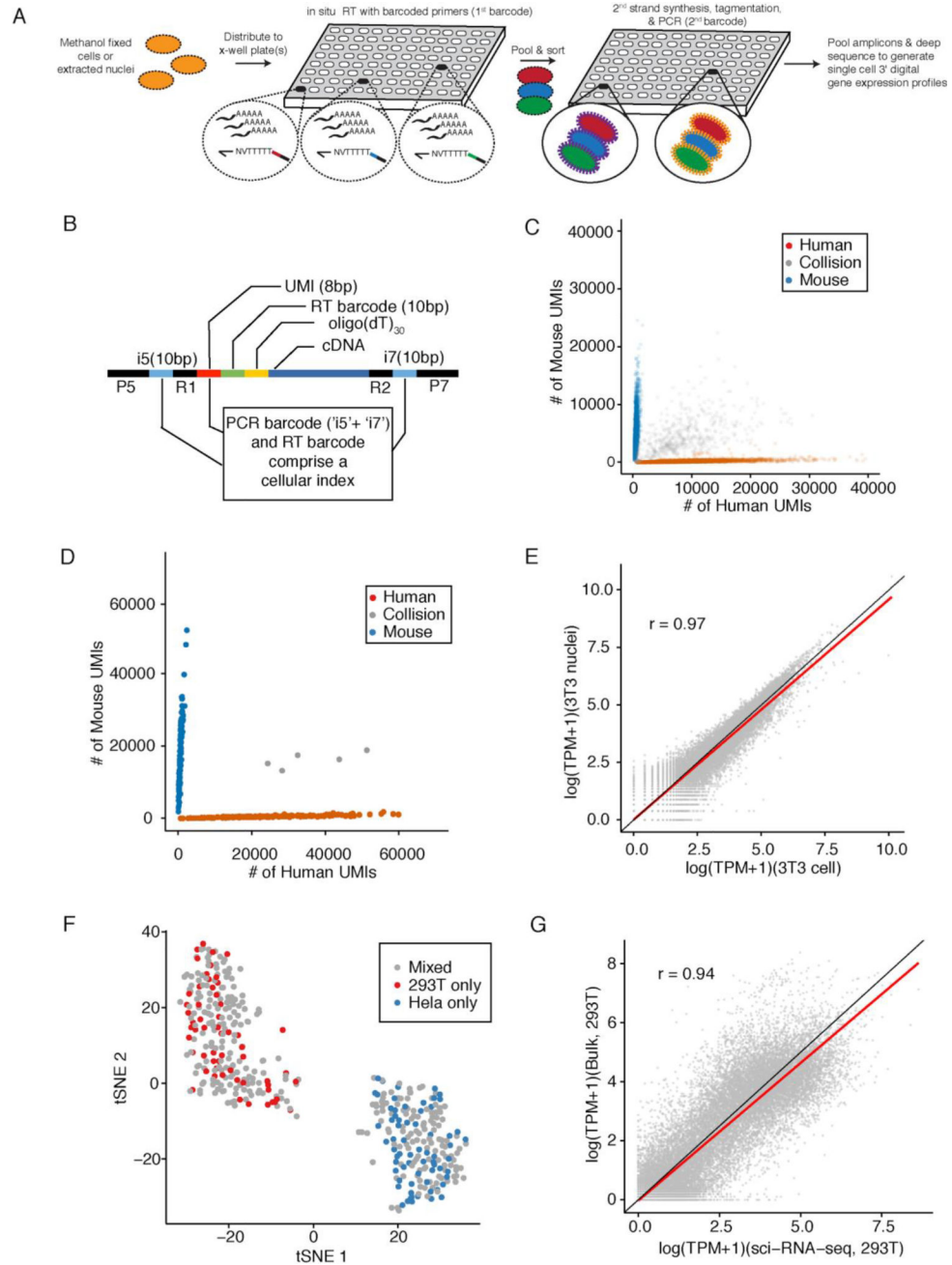


Fig. 1. sci-RNA-seq enables multiplex single cell transcriptome profiling

(A) Schematic of sci-RNA-seq workflow. (B) Schematic of sci-RNA-seq library amplicons. Index2 and read1 covers the i5 index, UMI and RT barcode. Index1 and read2 covers the i7 index and cDNA fragment. (C) Scatter plot of unique human and mouse UMI counts from 384×384 sci-RNA-seq. Blue: inferred mouse cells ($n = 5953$). Red: inferred human cells ($n = 3967$). Grey: collisions ($n = 884$). (D) Scatter plot of unique human and mouse cell UMI counts from 96×96 sci-RNA-seq with optimized protocol. Blue: inferred mouse cells ($n = 129$). Red: inferred human cells ($n = 160$). Grey: collisions ($n = 5$). In (C) and (D), only cells originating from wells containing mixed human and mouse cells are shown. (E) Correlation

between gene expression measurements in aggregated sci-RNA-seq profiles of NIH/3T3 cells (n = 238) vs. nuclei (n = 124). **(F)** tSNE plot of cells originating in wells containing HEK293T (red) (n = 60), HeLa S3 (blue) (n = 69) or a mixture (grey) (n = 321). **(G)** Correlation between gene expression measurements from aggregated sci-RNA-seq data vs. bulk RNA-seq data from a related protocol (33). (E) and (G) include linear regression (red) and $y=x$ (black) lines.

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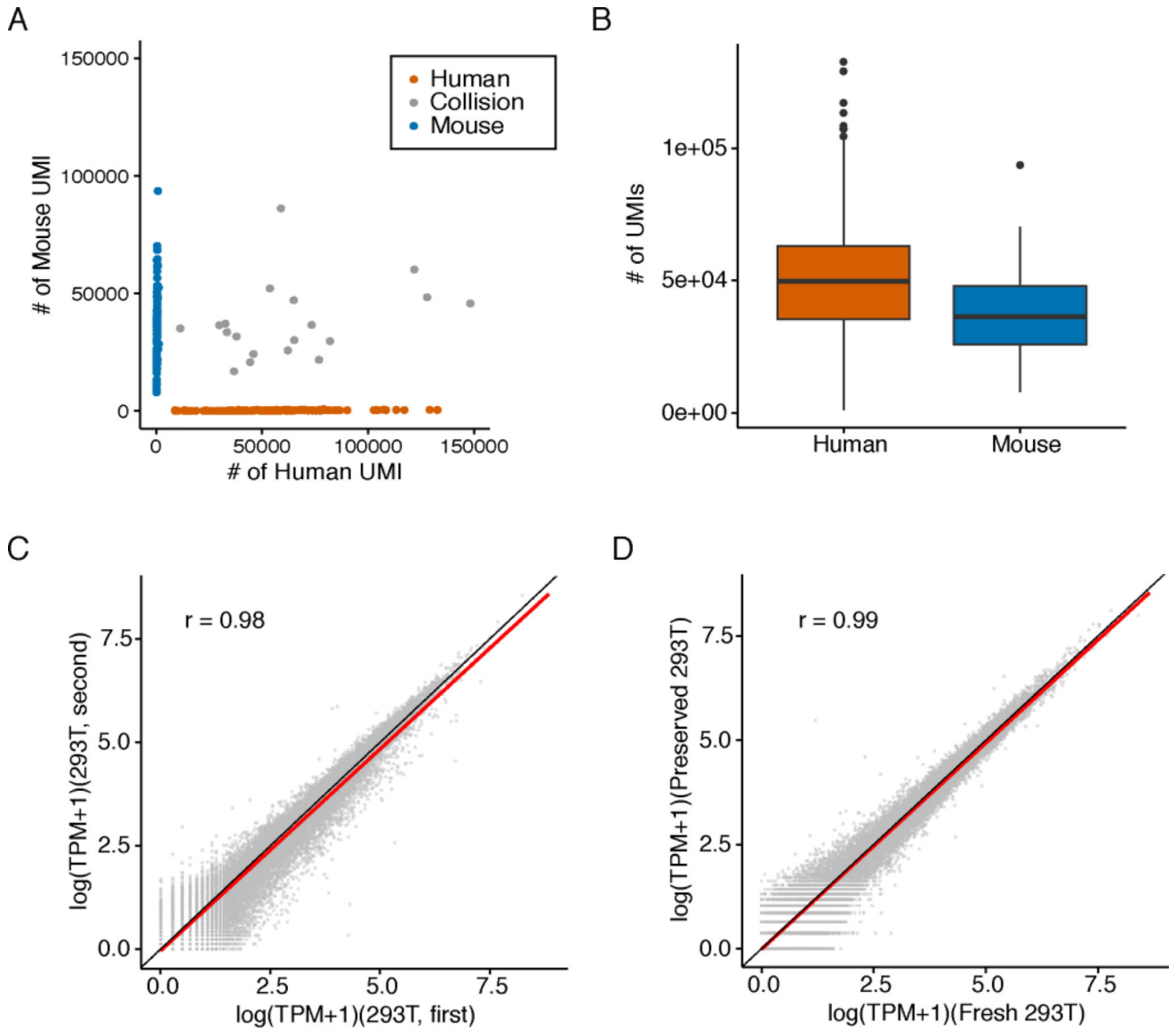


Fig. 2. sci-RNA-seq shows robust gene expression measurements

(A) Scatter plot of unique human and mouse UMI counts from a 16×84 sci-RNA-seq experiment on mixed HEK293T and NIH/3T3 cells (Table S1). Blue: inferred mouse cells ($n = 109$). Red: inferred human cells ($n = 168$). Grey: collisions ($n = 19$). (B) Boxplots showing number of UMIs detected per cell. (C) Correlation between gene expression measurements in aggregated sci-RNA-seq profiles from experiments performed two months apart on independently grown and fixed cells. (D) Correlation between gene expression measurements in aggregated sci-RNA-seq profiles of fixed-fresh vs. fixed-frozen cells. (C) and (D) include linear regression (red) and $y=x$ (black) lines.

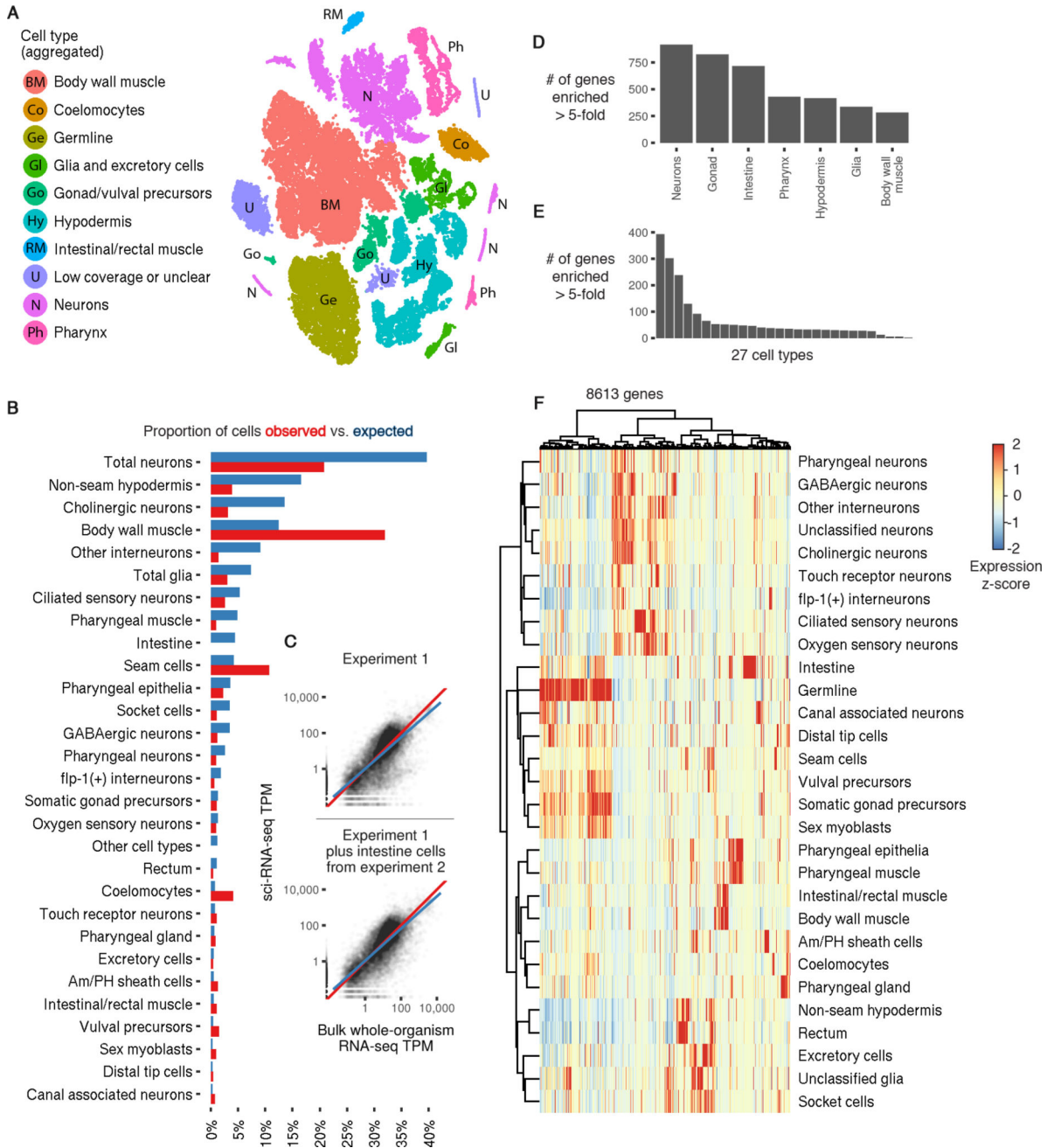


Fig. 3. A single sci-RNA-seq experiment highlights the single cell transcriptomes comprising the *C. elegans* larva

(A) t-SNE visualization of the high-level cell types identified. (B) Bar plot showing the proportion of somatic cells profiled in the first sci-RNA-seq *C. elegans* experiment that could be identified as belonging to each cell type (red) compared to the proportion of cells from that type present in an L2 *C. elegans* individual (blue). (C) Scatter plots showing the log-scaled transcripts per million (TPM) of genes in the aggregation of all sci-RNA-seq reads (x axis) or in bulk RNA-seq (y axis; geometric mean of 3 experiments). Top plot includes only the first sci-RNA-seq experiment. Bottom plot also includes intestine cells

from the second sci-RNA-seq experiment. **(D)** Number of genes that are enriched at least 5-fold in a specific tissue relative to the 2nd-highest-expressing tissue, excluding genes for which the differential expression between the 1st and 2nd-highest expressing tissues is not significant (q -value > 0.05). **(E)** Same as **(D)** except comparing cell types instead of tissues. **(F)** Heatmap showing the relative expression of genes in consensus transcriptomes for each cell type estimated by sci-RNA-seq. Genes are included if they have a size-factor-normalized mean expression of >0.05 in at least one cell type (8,613 genes in total). The raw expression data (UMI count matrix) is log-transformed, column centered and scaled (using the R function `scale`), and the resulting values are clamped to the interval $[-2, 2]$.

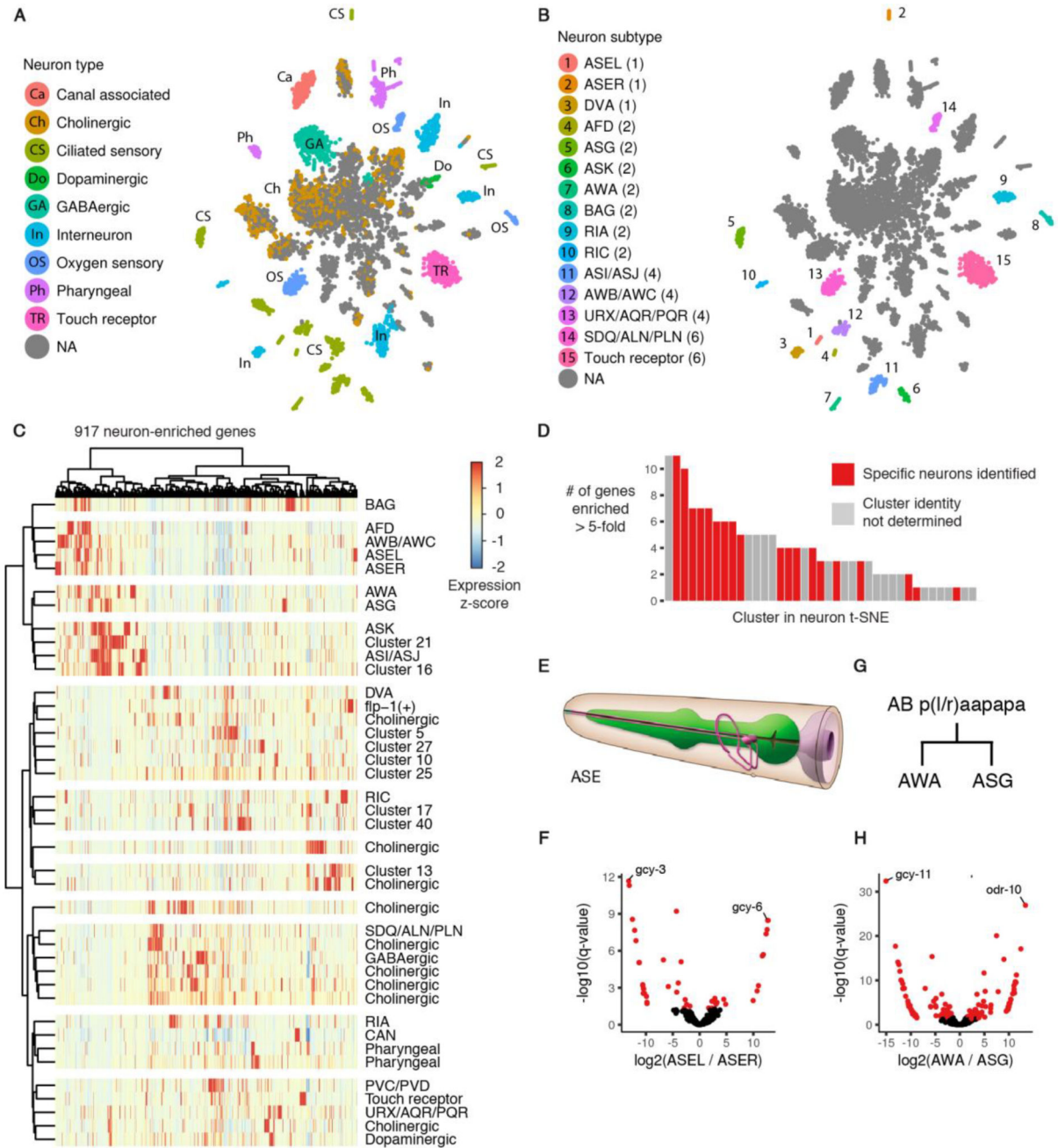


Fig. 4. sci-RNA-seq reveals the transcriptomes of fine-grained anatomical classes of *C. elegans* neurons

(A) t-SNE visualization of high-level neuronal subtypes. Cells identified as neurons from the t-SNE clustering shown in Fig. 3A were re-clustered with t-SNE. (B) Clusters in the neuron t-SNE that can be identified as corresponding to one, two, or four specific neurons in an individual *C. elegans* larva. The number of neurons of each type are shown in parentheses. (C) Heatmap showing the relative expression of neuron-enriched genes across 40 neuron clusters identified by t-SNE and density peak clustering. Genes are included if their expression in the aggregate transcriptome of all neurons in our data is >5-fold higher than

their expression in any other tissue, excluding cases where the differential expression is not significant (q-value > 0.05). **(D)** Distribution for each neuron cluster of the number of genes that are expressed >5-fold higher in that cluster than in the 2nd-highest expressing neuron cluster (q-value for differential expression < 0.05). **(E)** Cartoon illustrating the position of the left and right ASE neurons (pink) relative to the pharynx (green); reproduced with permission from www.wormatlas.org (60). **(F)** Volcano plot showing differentially expressed genes between the left and right ASE neurons. Points in red correspond to genes that are differentially expressed (q-value < 0.05) with a > 3-fold difference between the higher- and lower-expressing neuron(s). **(G)** The left AWA and ASG neurons arise from the embryonic cell AB plaapapa; the right AWA and ASG neurons arise from AB praapapa. **(H)** Volcano plot showing differentially expressed genes between the AWA and ASG neurons.

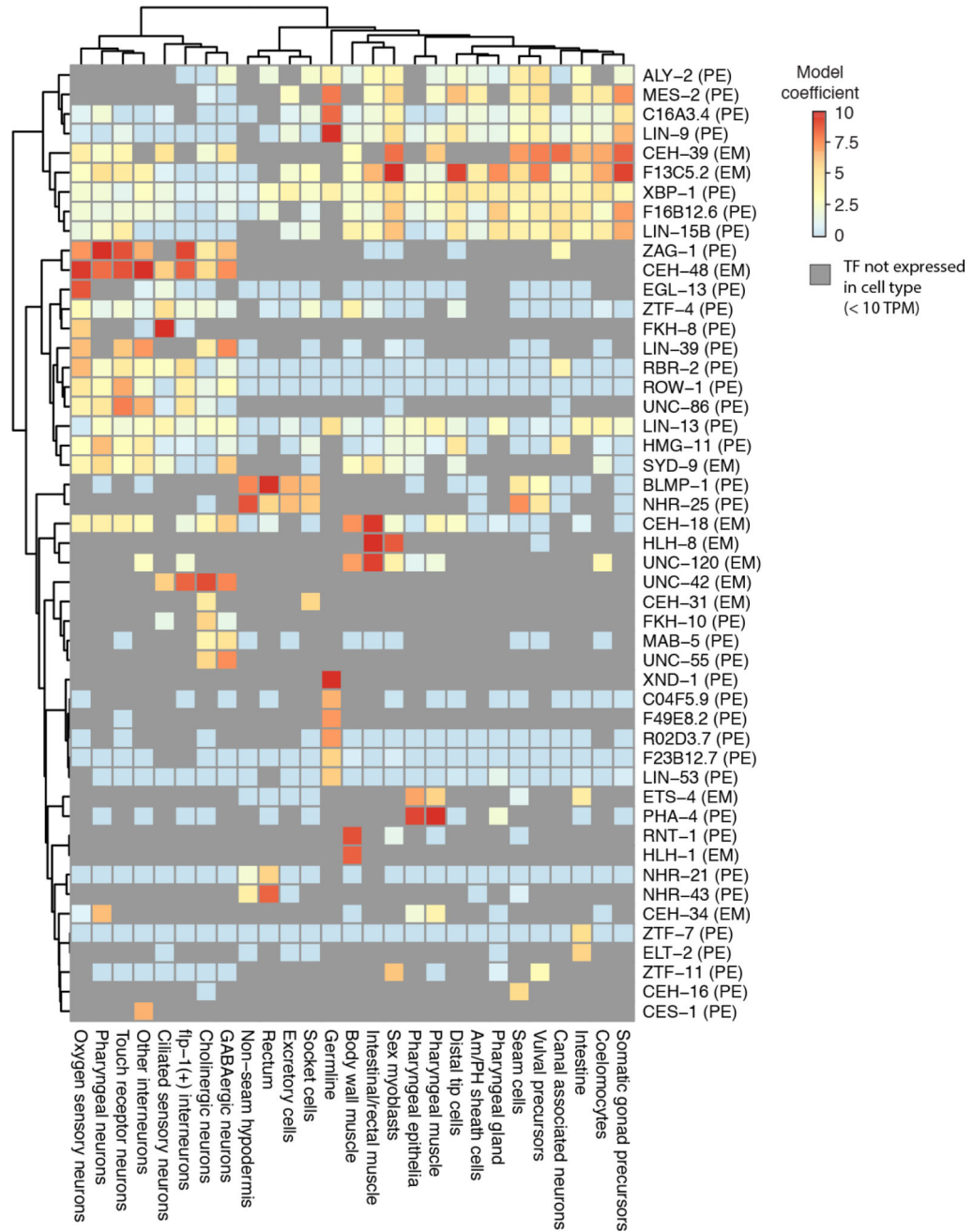


Fig. 5. Cell type specific expression profiles from sci-RNA-seq enable the deconvolution of whole-animal transcription factor ChIP-seq data

For each of 27 cell types, a regularized regression model was fit to predict log-transformed gene expression levels in that cell type on the basis of ChIP-seq peaks in gene promoters (31). The ChIP-seq data was generated by the modENCODE (61) and modERN consortia (46), profiling transcription factor binding in whole *C. elegans* animals. “EM” next to a TF label indicates the ChIP-seq data for the TF is from an embryonic stage, while “PE” indicates the data is from a post-embryonic stage. Colors in the heatmap show the extent to which having a ChIP-seq peak for a given TF in a gene promoter correlates with increased expression in a given cell type. Peaks in “HOT regions” (31) are excluded. Grey cells in the

heatmap correspond to cases where a TF is not expressed in a cell type (< 10 TPM), in which case ChIP-seq data for that TF is not considered by the regression model.

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