AdRoit: an accurate and robust method to infer complex transcriptome composition

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Abstract

Bulk RNA sequencing technology provides the opportunity to understand biology at the whole transcriptome level without the prohibitive cost of single cell profiling. Advances in spatial transcriptomics enable to dissect tissue organization and function by genome-wide gene expressions. However, the readout of both technologies is the overall gene expression across potentially many cell types without directly providing the information of cell type constitution. Although several in-silico approaches have been proposed to deconvolute RNA-Seq data composed of multiple cell types, many suffer a deterioration of performance in complex tissues. Here we present AdRoit, an accurate and robust method infer the cell composition from transcriptome data comprised of multiple cell types. AdRoit uses gene expression profile obtained from single cell RNA sequencing as a reference. It employs an adaptive learning approach to correct the sequencing technique difference between the single cell data and the bulk or spatial transcriptome data, enabling cross-platform readout comparability. Our

systematic benchmarking and applications, which include deconvoluting complex mixtures that encompass 30 cell types, demonstrate its superior sensitivity and specificity compared to other existing methods as well as its utilities. In addition, AdRoit is computationally efficient and runs orders of magnitude faster than many existing methods.

Introduction

RNA sequencing is a powerful tool to understand the biology of normal and disease tissues at the whole transcriptome level. It helps to elucidate disease mechanism and identify potential treatment targets¹. Due to the presence of heterogeneous cell populations, the bulk tissue transcriptome only characterizes the overall gene expression across multiple cell types. The cell type identifies and their prevalence remain unknown in the bulk data. However, knowledge of the cell type composition is often critical to understand the biology. For instance, the constitution of stromal and immune cells sculpts the tumor microenvironment, which is essential in cancer progression or response to immune check point inhibition^{2–6}. Excessive expression of cytokines in particular leukocyte types underlines the etiology of many chronic inflammatory diseases^{7–11}. Such information cannot be directly read out from the bulk RNA-Seq.

Recent breakthroughs in spatial transcriptomics methods enable characterizing whole transcriptome-wise gene expressions at spatially resolved locations in a tissue section¹². However, it remains challenging to reach a single cell resolution while measuring tens of thousands of genes transcriptome-wise. Some widely used technologies can achieve a resolution of 50-100 μm , equivalent to 3–30 cells depending on the tissue type^{12,13}. The

transcripts therein may originate from more than one cell type, resulting in another form of RNA-Seq data of multiple cell types. Unlike the bulk RNA-Seq, the profiling data at each spot contains substantial dropouts as merely a few cells are sequenced, imposing additional challenges to demystify the cell type content. We refer to bulk RNA-Seq data and spatial transcriptome data at the multi-cell resolution as compound RNA-Seq data hereafter.

The rapid development of single-cell RNA-Seq (scRNA-Seq) technologies has allowed for cell-type specific transcriptome profiling¹⁴. It provides the information missing from the RNA-Seq data of tissues comprised of multiple cell types (e.g., bulk RNA-Seq). Nevertheless, the technologies have low sensitivity and substantial noise due to the high dropout rate and the cell-to-cell variability. Consequently, scRNA-Seq technologies require a large number of cells (thousands to tens of thousands) to ensure statistical significance in the results. In addition, the cells must remain viable during the capture. These requirements render the scRNA-Seq technologies costly, prohibiting their application in clinical studies that involve many subjects or cannot allow real time tissue dissociation and cell capture. Furthermore, scRNA-Seq technologies may not be well suited to characterizing cell-type proportions in solid tissues because the dissociation and capture steps may not have the same efficiency for different cell types^{15–17}.

Because sequencing at the single cell level is not always feasible and also for the purpose of better interpreting many highly valuable existing bulk RNA-Seq or spatial transcriptomic data sets, in silico approaches have been developed to infer cell type proportions from compound

RNA-Seq data^{18–24}. The most common strategy is to conduct a statistical inference through the maximum likelihood estimation (MLE)²⁵ or the maximum a posterior probability estimation (MAP)²⁶ on a constrained linear regression framework, wherein the unobserved mixing proportion of a finite number of cell types are part of the latent variables to be optimized^{19,21–24}. The deconvolution methods are often applied to dissect the immune cell compositions in blood samples^{27–31}. However, their performance in more complex tissues, such as the nervous, ocular, respiratory or gastrointestinal organs, remains unclear. These tissues often contain many cell types (10^1 – 10^2) and the difference among related cells can be subtle, rendering the deconvolution a challenging task. For example, a recent study on the mouse nervous system using scRNA-Seq found more than 200 cell clusters and many are highly similar neuronal subtypes³².

Earlier works often utilized the transcriptome profiling of the purified cell populations to estimate the gene expressions of cell type (e.g. Cibersort)¹⁹. More recently, acquiring cell type specific expression from the scRNA-Seq data was shown to be an intriguing alternative^{21–24}. Although it provides higher throughput by measuring multiple cell types in one experiment, profiling at the single cell level has a high noise floor. The accuracy of deconvolution using scRNA-Seq data as the reference may be affected by the data noise if not treated properly. Moreover, the platform difference between the compound data and the single cell data cannot be ignored.

To overcome these challenges, additional information from the data may be considered. A recent method that weighs genes according to their expression variances across samples greatly improved the accuracy²², highlighting the importance of gene variability in inferring cell type composition. Some other methods and applications have pointed out the importance of cell type specific genes^{24,28,31,33}. In these works, the cell type specific expression was only used to select the input genes (e.g., markers). Nonetheless, it measures how informative a gene is in distinguishing cell types and thus can be incorporated as a part of the model. To address the platform difference between the compound data and the single cell data it is usually assumed there exists a single scaling factor or a linearly scaled bias for all genes that can be learned and corrected accordingly^{22,23}. This assumption does not hold, as the impact of changing platforms is different for each gene. Though learning a uniform scaling factor would correct the difference in most genes, a few genes that remain significantly biased can easily confound the estimation, especially under a linear model framework. Thus, a gene-wise correction should be considered.

In this work, we present a new deconvolution method, AdRoit. A unified framework that jointly models the gene-wise technology bias, as well as the cell type specificity and cross-sample variability of genes. The method estimates the cell type constitution in the compound RNA-Seq samples using relevant single cell data as a training source. Genes used for deconvolution were automatically selected from the single cell data based on their information richness. Uniquely, it uses an adaptively learning approach to estimate gene-wise scaling factors, addressing the issue that each platform impact genes differently. AdRoit further makes use of regularization to avoid collinearity among closely related cell subtypes that are common in complex tissues. Over

a set of comprehensive benchmarking data with a varying cell composition complexity, AdRoit showed superior sensitivity and specificity to other existing methods. Applications to real RNA-Seq bulk data and spatial transcriptomics data revealed strong and expected biologically relevant information. We believe AdRoit offers an accurate and robust tool for cell type deconvolution and will enhance the value of bulk RNA-Seq and the spatial transcriptome profiling.

Results

Overview of the AdRoit framework

AdRoit estimates the proportions of cell types from transcriptome data of a mixed cell population including but not limited to bulk RNA-Seq and spatial transcriptome. It directly models the raw reads without normalization, preserving the difference in total amounts of RNA transcript in different cell types. The method utilizes, as a reference, the relevant pre-existing scRNA-Seq data with cell identity annotation. It selects informative genes, estimates the mean and dispersion of the expression of selected genes per cell type, and constructs a weighted regularized linear model to infer percent combinations (Fig. 1a). Because sequencing platform bias may impact genes differently^{15,34,35}, a uniform scaling factor for all genes does not sufficiently eliminate such bias. A key innovation of AdRoit is that it uniquely adopts an adaptive learning approach, where the bias is first estimated for each gene, then adjusted such that more biased genes are corrected with a larger scaling factor (Fig. 1b).

Fig. 1

AdRoit considers a comprehensive set of other relevant factors including the cross-sample variability and the cell type specificity of genes, as well as the collinearity of expression profiles among closely related cell types. The cross-sample variability of a gene is the variation of its expression in the same cell type across multiple bulk samples. It shall be distinguished from the expression change in the gene over different cell types. AdRoit decreases the weight of genes with high cross-sample variability whilst increasing the weight of those with an expression highly specific to certain cell types. The definition of cross-sample variability and cell type specificity also accounts for the overdispersed nature of counts data. Lastly, AdRoit adopts a linear model to ensure the interpretability of the coefficients. At the same time, AdRoit includes a regularization term to minimize the impact of statistical collinearity. Each of the factors

contributes an indispensable part to AdRoit, leading to an accurate and robust deconvolution method for inferring complex cell compositions.

To evaluate the performance, we compared AdRoit with NNLS^{18,36}, MuSiC²², Bisque³⁷, and SPOTlight³⁸ for bulk data deconvolution, and Stereoscope²³, Cell2location³⁹, and SPOTlight³⁸ for spatial transcriptomics data deconvolution. When evaluating the algorithms, a common practice is to pool the single cell data to synthesize a “bulk” sample with the known ground truth of the cell composition. We measured the performance by comparing the estimated cell proportions with true proportions using four metrics: mean absolute difference (mAD), root mean square deviation (RMSD), and two correlation statistics (i.e., Pearson and Spearman). While Pearson reflects linearity, Spearman measures whether the estimated results and the ground truth are monotonically related, even if their relationship is not linear, which avoids the artificial high linearity scores driven by outliers when the majority of estimates are small. Thus, both correlations statistics were included. Good estimations feature low mAD and RMSD along with high correlation statistics. When estimating cell proportions for a synthetic sample, cells from this sample were excluded from the input single cell reference (i.e., leave-one-out) to avoid overfitting. We further applied AdRoit to real bulk RNA-Seq data and validated the results by available RNA fluorescence *in-situ* hybridization (RNA-FISH) data. The estimates were further confirmed by the biological knowledge of human pancreatic islets. We also used AdRoit to map cell types within spatial spots, and the accuracy was verified by *in-situ* hybridization (ISH) images from Allen mouse brain atlas⁴⁰.

AdRoit excels in both simple and complex cell constitutions

We started with a simple human pancreatic islets data set that contains 1492 cells and four distinct endocrine cell types (Alpha, Beta, Delta, and PP cells)⁴¹ (Supplementary Fig. 1a; Supplementary Table 1). The synthesized bulk data were constructed by mixing the single cell data at known proportions. All methods except SPOTlight achieved satisfactory performance according to the evaluation metrics, AdRoit performed the best as reflected by scatterplots of estimated proportion vs. true proportion (Supplementary Fig. 1b, Supplementary Table 2). It had the lowest mAD and RMSD, and the highest correlation values (MuSiC's correlations are comparable) among the five methods tested (Supplementary Fig. 1c). Given that MuSiC is a weighted NNLS and consistently showed an improved accuracy here and in the original publication²², we excluded NNLS from further evaluation hereafter.

We then tested the methods on a complex tissue - human trabecular meshwork (TM). We acquired published single cell data that contains 8758 cells and 12 cell types from 8 donors⁴². The data include 3 similar types of endothelial cells, 2 types of Schwann cells, and 2 types of TM cells (Supplementary Fig. 2; Supplementary Table 3). Cells from each donor were pooled as individual synthetic bulk samples. The cell type proportions vary from <1% to 43%. These proportions were the ground truth cell composition and were compared head-to-head with the estimated proportions inferred by four methods. For each synthetic bulk sample, estimations were performed using a reference built from cells of remaining donors (i.e., leaving-one-out). In each of the 8 samples, the estimates made by AdRoit best approximated the true proportions. In particular, AdRoit had the lowest mAD (0.016) and RMSD (0.025), and the highest correlation

values (Pearson = 0.97; Spearman = 0.94) among the methods (Fig. 2a). We further assessed the deviation of the estimates from the true proportions for each cell type. AdRoit consistently had the lowest deviations from the true proportions for all cell types, as well as the lowest variation among 8 samples (Fig. 2b, blue dots), indicating robustness over various cell types and samples (Supplementary Fig. 3, Supplementary Table 4).

Fig. 2

AdRoit has better sensitivity and specificity

To assess the level of granularity that AdRoit can achieve when deconvoluting similar cell types, we used well-studied, closely related immune cells from myeloid lineage and lymphoid lineage. We obtained B cells, naïve CD4⁺ T cells, memory CD4⁺ T cells, CD8⁺ T cells, natural killer (NK) cells, dendritic cells (DC), CD14⁺ monocytes, FCGR3A⁺ monocytes, and platelets from a public release of the human peripheral blood mononuclear cell (PBMC) single cells by 10x Genomics (see also Data Availability). We simulated bulk samples that each contained a mixture of

201 myeloid lineage or lymphoid lineage cell types. Such a sample was created by randomly
 202 selecting cells from a given set of cell types and mixing them according to their predefined
 203 percentages. We repeated this procedure 100 times for a series of mixing proportions among
 204 myeloid or T lymphoid cells. The bulk mixtures of CD14⁺ monocytes, FCGR3A⁺ monocytes and
 205 dendritic cells (DC) followed three schemes of proportions: 0.33:0.33:0.33 (mix0), 0.1:0.45:0.45
 206 (mix1) and 0.1:0.3:0.6 (mix2). The same ratios were applied to the mixtures of naïve CD4⁺ T,
 207 memory CD4⁺ T and CD8⁺ cells. All the nine cell types in the PBMC data were used to build the
 208 reference for the deconvolution. AdRoit accurately estimated the known percentages in all
 209 cases (Fig. 3a, Supplementary Table 5). In addition, there were no noticeable false positive
 210 predictions of any excluded cell types. These results highlight that AdRoit can capture the fine
 211 difference among similar cell types and quantitatively distinguish them.

223

224 We designed a simulation to test sensitivity and specificity. we selected 6 out of the 12 human

225 trabecular meshwork cell types, i.e., Schwann-cell like cell, TM1, smooth muscle cell,

226 melanocyte, macrophage, and pericyte, from each donor sample and pooled them within that

227 sample to synthesize 8 new bulk samples. The unselected 6 cell types were considered absent

228 in the bulk samples. Some cell types selected highly resembled those in absence, challenging

229 the programs to pinpoint the right cell type present in the bulk among similar candidates. We

230 provided the full list of 12 single cell types as the reference to the programs to estimate the cell

231 type proportions. Consistently across 8 samples, AdRoit had the most accurate estimates for

232 the 6 present cell types, and 0 or close to 0 estimated values for the non-existing cell types in

233 the simulated bulk data. MuSiC, Bisque and SPOTlight were notably less accurate on the 6

234 selected cell types, as well as they had many non-negligible values for the 6 cell types excluded

235 in the 8 synthetic samples (Fig. 3b, Supplementary Table 6). For example, smooth muscle cells

236 accounted for ~14% in donor 4 but were largely missed (~0.03%) by MuSiC. We noted that TM2

237 had false non-zero estimates from all methods though not included. This is because TM2 is

238 easily mistaken as TM1 due to their high similarity⁴². Nonetheless, AdRoit's estimates of TM2

239 were consistently low across samples (<1% for 44 out of 48 estimates), while the other

240 methods had significantly larger estimates of TM2 that occasionally even exceeded the TM1

241 estimates. For a systematic comparison, we constructed the receiver operating characteristic

242 (ROC) curve by varying the threshold of detection (i.e. a cutoff below which the cell type was

243 deemed undetected) (Fig. 3c). AdRoit had a significantly higher area under the curve (AUC) than

the other methods (AdRoit: 0.95, Bisque: 0.79, MuSiC: 0.74, SPOTlight: 0.37), implying both better sensitivity and specificity.

As complex tissues often contain several tens of different cell types, we continued the evaluation of the sensitivity and specificity in more complicated cell mixtures. We utilized the published mouse brain single cell atlas by Zeisel et al that contains a comprehensive set of neuronal and supporting cells³². Without loss of generality, we consolidated the original annotation into 46 major cell types (Methods, Supplementary Table 7). To synthesize a bulk mixture, we randomly selected 30 cell types and pooled all their associated cells. This procedure was repeated independently 100 times for thorough coverage of different cell type combinations. The deconvolution was performed using all 46 cell types as the reference. As illustrated in Fig. 3d, AdRoit was able to estimate the proportions closely consistent with the ground truth while the other three tools made less satisfactory predictions (see also Supplementary Table 8). The ROC curve further indicates that AdRoit has the best sensitivity and specificity given its highest AUC (Fig. 3e; AdRoit: 0.99, Bisque: 0.77, MuSiC: 0.80, SPOTlight: 0.67). These observations convince us that AdRoit offers a leading performance on highly complex cell constitutions.

AdRoit outperforms in deconvoluting closely related cell subtypes

To further evaluate AdRoit when multiple highly homologous subtypes of cells are present in a complex tissue, we performed scRNA-Seq experiment on mouse lumbar dorsal root ganglion (DRG) from five mice. Following the standard analysis pipeline (Methods), we obtained 3352

single cells after quality control procedures. After clustering and annotation, we discovered 14 cell types including multiple subtypes of neuronal cells (Fig. 4a, Supplementary Table 9). The heatmap of the top marker genes showed distinct patterns of the major cell types as well as similar patterns of the subtypes (Supplementary Fig. 4a), with the cell type proportions varying from 0.5% to 33.71% (Supplementary Fig. 4b). These 14 cell types include 3 subtypes of neurofilament containing neurons (i.e., NF_Calb1, NF_Pvalb, NF_Ntrk2.Necab2), 3 subtypes of non-peptidergic neurons (i.e., NP_Nts, NP_Mrgpra3, NP_Mrgprd), and 5 subtypes of peptidergic neurons (i.e., PEP1_Dcn, PEP1_S100a11.Tagln2, PEP1_Slc7a3.Sstr2, PEP2_Htr3a.Sema5a, PEP3_Trpm8). Also discovered were tyrosine hydroxylase containing neurons (Th), satellite glia, and endothelial cells. Such complex compositions formed a challenging testing ground for evaluating the ability to distinguish closely related cell types. We again performed the leave-one-out deconvolution on five synthesized bulk samples.

Fig. 4

AdRoit had highly accurate estimations on all cell subtypes across samples (Fig. 4b). Worth noting, for the rare cell types that account for less than 5%, AdRoit still had a good estimation that is close to the true proportions and never missed a single cell type, showing that AdRoit is very robust on rare cell types. For example, 0.51% endothelial cells were predicted to be 0.35%, and 1.05% NF2_Ntrk2.Necab2 cells were predicted to be 0.85% (Supplementary Fig. 5, Supplementary Table 10). Conversely, Bisque, MuSiC, and SPOTlight were notably less accurate, especially for the cell types less than 5%, and all three missed multiple cell types including some large clusters accounting for ~10% of the cells (PEP1_Slc7a3.Sstr2 cells of Sample5). We continued to examine how much the variability of the estimates was in each sample. We computed the same 4 metrics used previously to evaluate the performance on each of the 5 synthetic samples and compared them head-to-head among the algorithms. This fine comparison showed AdRoit significantly outperformed on nearly every sample (Fig. 4c). Moreover, the performance metrics of AdRoit were highly consistent across samples with the lowest variability among all four methods.

AdRoit excels on spatial transcriptomics data

Given the promising performance on complex tissues, we moved forward to test AdRoit's applicability to spatial transcriptome data. Spatial transcriptomics data differs from bulk RNA-Seq data in that each spot only contains transcripts from a handful of cells (3-30)¹². Some of the spots contain multiple cells of the same type, while others may have heterogeneous cell types at varying mixing percentages (e.g., spatial spots at the boundary of different cell types). As the spot is a pool of only a few cells, the variations across spatial spots are expected to be greater

than the changes in bulk samples, imposing an additional challenge for deconvolution. We simulated a large number of spatial spots (3200 in total) by using sampled cells from the DRG single cell data above (Methods), then compared AdRoit with Stereoscope, Cell2location, and SPOTlight over a range of simulation scenarios.

We first tested whether the methods could correctly infer a single cell type when the spots contain cells from that same type. For each of the 14 cell types from DRG, we sampled 10 cells and pooled them to form a spatial spot. We repeated the simulation 100 times for robust testing, then used the full set of 14 cell types as reference to deconvolute the 1400 simulated spots. All methods except SPOTlight were able to identify the correct cell types with high accuracy on the target cell types (i.e., percentages close to 1) and comparably low estimated values (i.e., percentages close to 0) for other cell types excluded from the simulation(Supplementary Fig. 6, Supplementary Table 11).

We then proceeded to a more difficult scenario where we sampled cells from the 5 PEP neuron subtypes and mixed them. We created three simulation schemes for a comprehensive evaluation: 1) 5 PEP subtypes had the same percent of 0.2; 2) PEP1_Dcn was 0.1 and the other 4 were 0.225; 3) PEP1_S100a11.Tagln2 and PEPE1_Dcn were 0.1, PEP2_Htr3a.Sema5a and PEP1_Slc7a3.Sstr2 were 0.2, and PEP3_Trpm8 was 0.4. Again, each simulation scheme was repeated 100 times. Under each scheme, the estimates by AdRoit consistently centered around the true proportions while the predictions of irrelevant cell types remained close to 0 (Fig. 5a, Supplementary Table 11). In comparison, Cell2location systematically overestimated the

PEP1_Slc7a3.Sstr2 by about 2.5%. Stereoscope more notably overestimated
 PEP2_Htr3a.Sema5a and underestimated the PEP3_Trpm8. SPOTlight had high noise for some
 cell types (e.g., endothelial and satellite glia) and generally deviated from the truth for multiple
 cell types under all simulation schemes.

Fig. 5

We expanded the simulated spatial spots to the mixture of 3 NP cell subtypes and the mixture
 of 3 NF subtypes. In addition, we blended either NP_Mrgpr3 cells, NF_Cab1 cells, or
 PEP3_Trpm8 cells with three other cell types (i.e., Th, satellite glia and endothelial). For all
 these simulated spatial spots, AdRoit's estimates consistently centered around the true

proportions, whereas the other methods deviated by different degrees in most of the simulated schemes (Supplementary Fig. 7, Supplementary Table 11). We speculate one important reason for the underperformance of some methods such as Stereoscope is that they normalize the total UMI counts to the same value for all cells. However, in real world, different cell types unlikely have the same amount of RNA transcripts (e.g. immune cells have about 10 fold fewer total UMIs than the neurons). Our simulation pooled the cells by adding up the raw UMI counts per gene, which we believe best mimics the real data. Normalizing the data by total UMI not only eliminates this factual difference between cell types but may distort the data from the assumed negative binomial distribution. AdRoit avoids this problem by model the raw UMI counts.

Next, we asked how sensitive the methods are in detecting rare cell populations. We simulated mixtures of 3 PEP subtypes (i.e., PEP1_Slc7a3.Sstr2, PEP2_Htr3a.Sema5a, and PEP3_Trpm8) wherein the percentage of PEP3_Trpm8 cells varied from 0.01 to 0.1 by 0.01, and the other two cell types sharing the remaining percentage equally (Methods). At each given mixing ratio, the simulation was repeated 100 times. We then checked how accurately the concentration of PEP3_Trpm8 cells was estimated. The medians of AdRoit's estimates were closest to the true proportions (Fig. 5b, red dashed lines) among all 4 methods, followed by Cell2location. Stereoscope's estimates were systematically lower than the true values and failed to detect the PEP3_Trpm8 cells when the simulated proportion was below 0.06. On the other hand, SPOTlight constantly overestimated the presence of PEP3_Trpm8 cells. The median of its predictions remained around 9% even when the true population was at 1%. Such observations

are consistent with the frequent false positives made by SPOTlight when deconvoluting the simulated bulk samples (Fig. 3b). This comparison implied AdRoit is more advantageous in detecting low percent cells. For a complete evaluation, we replicated the comparison using 5 additional sets of cell mixtures: NF_Calb1 with NF_Pvalb and NF2_Ntrk2.Necab2 (NP subtypes); NP_Mrgpra3 with NP_Mrgprd and NP_Nts (NP subtypes); NF_Calb1 with Th, satellite glia and endothelial (NF_Calb1 + others); NP_Mrgpra3 with Th, satellite glia and endothelial (NP_Mrgpra3 + others); and PEP_Trpm8 with Th, satellite glia and endothelial (PEP_Trpm8 + others). In each set, the first cell type mentioned was considered rare with its percent varied from 0.01 to 0.1. The rest cell types shared the remaining proportion evenly. At each given percent of the rare cell type, we computed how many times out of 100 the rare cell component was detected (estimation > 0.005). AdRoit had systematically high detection rates especially when the percent is below 3%. (Fig. 5c, Supplementary Table 12). Note that the apparent high detection rates in SPOTlight were merely the result of its high false positive estimates (Fig. 3b and Fig. 5b). The detection sensitivity of Cell2location was comparable to that of AdRoit. Notably, given a rare cell population of 5%, both AdRoit and Cell2location achieved a detection rate >90% under all simulation schemes, making them powerful tools to uncover rare cells.

Application to real bulk RNA-Seq data of human pancreatic islets

Though using synthetic bulk data based on the mixing of single cells is a useful benchmarking strategy, the bulk and single cell RNA-Seq often use distinct RNA library preparations and sequencing protocols. The capability of a method to deconvolute real bulk samples shall be addressed to ensure it is useful in real-world applications. We acquired 70 real human

pancreatic islets bulk samples from published studies^{41,43,44} (Supplementary Table 13) and used single cell data of the same tissue⁴¹ as the reference to infer the percentages of 4 endocrine cell types (i.e., Alpha, Beta, Delta, PP). The 70 bulk samples were collected from 39 distinct donors, including 26 healthy donors, and 13 donors with type 2 diabetes (T2D). Each donor contributed 1 to 5 bulk RNA sample repeats.

Replicates from the same donor are expected to have similar compositions and thus were used to assess the reproducibility of the estimates from AdRoit. For all cell types, AdRoit had consistent estimates for the same donors (Fig. 6a, Supplementary Table 14). The average standard deviations did not exceed 1% for all 4 cell types (i.e., Alpha: 0.010; Beta: 0.008; Delta: 0.004; PP: 0.002). To seek an independent validation, we obtained cell sorting results by RNA-FISH for 4 of the 39 donors⁴¹ (Supplementary Table 15). The estimated cell proportions in the 4 samples agreed well with the percentages measured by RNA-FISH (Fig. 6b). The consistency held for both major cells (Alpha and Beta) and the minor cells (Delta and PP). The observed reproducibility and the success of the independent validation showed AdRoit is reliable in deconvoluting real bulk RNA-Seq data.

Fig. 6

We then asked if AdRoit can detect known biological differences between healthy and T2D donors. Loss of functional insulin-producing Beta cells is a prominent characteristic of T2D^{45–47}, typically reflected by an elevated level of hemoglobin A1c (HbA1c)^{48,49}. Among the healthy donors, most Beta cell proportions estimated by AdRoit ranged from 50% to 75% (Fig. 6c), agreeing with the known percent range of Beta cells in human islets tissue^{50,51}. A significant decrease in the estimated Beta cell proportions was seen in T2D patients (p value = 4.1e-6 by two-sided t-test). Further, a linear regression of estimated Beta cell proportions on HbA1c levels showed a statistically significant negative association (p value = 1.8e-6), showing that AdRoit adequately reflected the cell composition difference between healthy donors and T2D patients.

Application to mouse brain spatial transcriptomics

We lastly demonstrated an application to the real spatial transcriptomics data. Given that the molecular architecture of brain tissues has been well studied, for this evaluation, we chose a set of mouse brain spatial transcriptomics data generated by 10x genomics, containing 2703 spatial spots. The consolidated version of the aforementioned mouse brain single cell atlas³² was used as the reference for deconvolution (Methods).

The cell contents inferred by AdRoit per spot appear to accurately match the expected cell types at that location (Supplementary Fig. 8, Supplementary Table 16). For example, the three subtypes of cortex excitatory neurons each occupied a sub-area in the cerebral cortex region. As another example, the shape of the hippocampal region was delineated by the estimated percentages of dentate gyrus granule/excitatory neurons. As an independent validation, we checked the consistency between the estimated cell types with the *in-situ* hybridization (ISH) images from Allen mouse brain atlas⁵². We chose 4 genes highly expressed in 4 brain regions respectively, i.e., *Spink8* for the hippocampal field CA1; *C1ql2* for the dentate gyrus; *Clic6* for the choroid plexus; and *Synpo2* for the thalamus³². The spots enriched with the 4 indicative cell types, hippocampal CA1 excitatory neuron type 2, dentate gyrus granule neuron type 2, choroid plexus cell and thalamus excitatory neuron type 1, as mapped by AdRoit, precisely co-localized with the regions that showed a strong signal of the 4 marker genes on the ISH images respectively (Fig. 6d). This agreement confirmed that the spatial mapping of cell types by AdRoit is reliable.

Computational efficiency

Besides the accuracy and robustness, another major advantage of AdRoit is its magnitude higher computational efficiency. AdRoit uses a two-step procedure to do the inference. The first step prepares the reference using the single cell data where the per-gene means and dispersions are estimated, and cell type specificity is subsequently computed. The built reference can be saved and reused. We tested the running time on the reference construction using the mouse brain single cell dataset discussed earlier that contains ~15,000 cells. It took about 4.5 minutes on a CPU that has 24 cores (23 used for parallel computing). The second step inputs the built reference and the compound RNA-Seq data and does the estimation. Deconvoluting ~2700 RNA-Seq samples of mixed cell populations took around 5 minutes. Therefore, AdRoit in total took less than 10 minutes and ~3Gb memory usage on a regular CPU. As a comparison, MuSiC and Bisque took about 1 hour and 30 minutes on the same data using the same CPU setup. SPOTlight took about 2 hours with the default parameters (cl_n = 100, hvg = 3000, ntop = 200, transf = "uv", method = "nsNMF", min_cont = 0). Stereoscope ran about 24 hours continuously with the published parameter setting (-scb 256 -sce 75000 -topn_genes 5000 -ste 75000 -lr 0.01 -stb 100 -scb 100) on a powerful V100 GPU with 80 cores and 16G memory. The efficiency of Cell2location was comparable to Stereoscope, taking about 18 hours on the V100 GPU (posterior n_sample = 1000, n_iteration = 10000, selection_specificity = 0.45). These methods can be prohibitive when seeking a quick turnaround.

Discussion

447 In this work we have demonstrated that AdRoit is capable of deconvoluting the cell
 448 compositions from the compound RNA-Seq data with a leading accuracy, measured by the
 449 consistency between the true and predicted cell proportions. Its advantage over the existing
 450 state-of-the-art methods was verified over a wide range of use cases. In particular, AdRoit
 451 excelled in complex tissues composed of more than ten different cell types with a wide range of
 452 cell proportions (e.g., trabecular meshwork, mouse brain and dorsal root ganglion). In these
 453 cases, AdRoit performed significantly better than the comparators on deconvoluting bulk RNA-
 454 Seq data. AdRoit is also more accurate and sensitive than tools specifically designed for
 455 deconvoluting spatial transcriptomics spots, especially in detecting low percent rare cells.
 456 Previous benchmarking often assumed the types of cells in the synthetic bulk data are not more
 457 or less than the cell types collected in the reference, and thus the only unknown was the
 458 proportion of each cell type. This assumption may not hold. Missing existing cell types or false
 459 predictions of non-existing ones can hinder the utility of an algorithm. Thus, besides the overall
 460 accuracy, we also examined the sensitivity and specificity of the algorithms. We observed a
 461 superior sensitivity and specificity in AdRoit, an important leverage for its usage in practice.
 462
 463 The reference single cell data used by AdRoit came from different platforms, such as the 10x
 464 Genomics Chromium Instrument (the mouse dorsal root ganglion), and the Fluidigm C1 system
 465 (the human pancreatic islets data). AdRoit consistently exhibited excellent performance across
 466 all benchmarking datasets independent of their single cell sequencing technology platforms.
 467 More importantly, this statement holds not only for deconvoluting the synthesized bulk data,
 468 but also for the real bulk RNA-Seq data. The latter typically does not apply the unique molecular

barcoding and requires a significantly different cDNA amplification procedure from what is used in the single cell RNA-Seq (Methods). Moreover, the sequencing depth, read mapping and gene expression quantification are dissimilar as well. The fact that AdRoit accurately dissected the cell compositions in the real bulk samples based on the single cell reference data further supports its cross-platform applicability.

We attribute the power of AdRoit to its comprehensive modeling of relevant factors. Firstly, we think a common rescaling factor is insufficient to correct the platform difference between single cells and the compound data. Rather, the impact of platform difference on genes is quite different and not linearly scaled. Correcting such differences entails rescaling factors specifically tailored to each gene. AdRoit uses an adaptive learning approach to estimate such gene-wise correcting factors and does the correction in a unified model. In addition, the contribution of a gene in a cell type to the loss function is jointly weighted by its specificity and variability in a cell type, where specificity and variability are defined in a way accounting for the overdispersion property of counts data. Our observations over the multiple benchmarking data sets also show that the coexistence of similar cell types may have induced a collinearity condition that negatively impacted the regression-based methods developed by others. Being able to alleviate this problem gives AdRoit an edge. All these factors help AdRoit to distinguish similar cell clusters while remaining sensitive enough to separate rare cell types.

Technically, the input profiles of individual cell types to AdRoit need not come from the single cell RNA-Seq. Bulk RNA-Seq profiles of individual isolated cell types can be used as well.

Nevertheless, using single cell RNA-Seq data as the reference has a few key advantages. It is a high throughput approach wherein multiple cell types can be interrogated simultaneously. Prior knowledge of the cell types in presence as well as their specific gene markers are not required, which allows novel cell types to be identified. Although detection of lowly expressed genes has been a challenge for the single cell RNA-Seq, significant enhancements have been demonstrated. For example, the number of detectable genes currently can reach an order of 10,000 per cell and keeps improving⁵³. As AdRoit focuses on the informative genes whose expressions are generally high, the detection limit of the single cell RNA-Seq does not impose a significant drawback. Indeed, given the single cell reference profiles, AdRoit successfully deconvoluted the real bulk RNA-Seq data and spatial transcriptomics data. The results suggest that, besides enriching our understanding of the bulk transcriptome data, AdRoit can leverage the usage of the vast amount and continuously growing single cell data as well.

AdRoit is a reference-based deconvolution algorithm. A comprehensive collection of the possible cell components is important. However, completeness may not always be guaranteed. Even with the single cell acquisition that is independent of prior knowledge, rare and/or fragile cell types may not survive through the capture procedure and hence are excluded. It is also difficult to generate a solid reference profile for cells that are versatile from sample to sample (e.g., tumor cells). Currently AdRoit deals implicitly with the components unknown to the reference. If an unknown cell type reassembles one of the referenced ones, it may be considered as part of the known cell type and their joint population is predicted. Such an outcome is acceptable as treating two similar cell types as one is still biologically meaningful

although the resolution of the system may be compromised. If the unknown component is dissimilar to all the known ones, it will be ignored by AdRoit because its representative markers are unlikely among the top weighted genes associated with the known components. At the same time, the distinct component is expected to have a unique gene expression pattern and thus unlikely to interfere significantly with the gene expressions from the known cell types. Therefore, AdRoit essentially deconvolutes the relative populations among the known cell components. For example, AdRoit was able to correctly uncover the populations of 4 endocrine cell types from the human islet bulk data despite the absence of many other cell types such as macrophages, Schwann cells and endothelial cells in the input single cell reference²⁰. Although under such a circumstance, the absolute percentages of the cells remain obscure, we expect their relative proportions can be studied and valuable. A future improvement is to explicitly model the unknown cell types and estimate their percentages upon the signals in the compound data that cannot be explained by the contribution from the known components. AdRoit may also be coupled with other programs⁵⁴ to output the deconvoluted cell type specific transcriptome profiles. The inferred cell type proportions by AdRoit can serve as the input to the downstream program and benefit the outcome with the reliability and accuracy demonstrated in this work.

Methods

Gene selection

AdRoit selects genes that provide information about cell type identity, excluding non-informative genes that potentially introduce noise. There are two ways for selecting such

genes: 1) union of the genes whose expression is enriched in one or more cell types in the single cell UMI count matrix. These genes are referred to as marker genes; 2) union of the genes that vary the most across all the cells in the single cell UMI count matrix, referred to as the highly variable genes. For marker genes, we recommend selecting the top ~200 genes, ranked by fold change, from each cell type for resolving complex compound transcriptome data. Considering some genes may be enriched in more than one cell type, we further require selected markers presenting in no more than 5 cell types to ensure specificity. We also suggest selecting a minimum of 1000 total unique marker genes for an accurate estimation. If not satisfied, one may consider expanding the number of top genes and/or loosen the statistical significance cutoff.

AdRoit also offers the option to use highly variable genes. To avoid the selected highly variable genes being dominated by large cell clusters, AdRoit first balances the cell types in the single cell UMI count matrix by finding the median size (i.e., the number of cells) of all clusters, then samples cells from each cluster to make its size equal to the median. Next, AdRoit computes the variance of each gene across all the cells in the balanced single cell UMI matrix. The variance-stabilizing transformation (VST)⁵⁵ is applied to normalize the data prior to the variance calculation. Genes with the top 2000 largest variances are then selected.

In both ways, mitochondria genes are excluded as their expression does not have information of cell identity. The results shown in the current paper were based on the marker genes derived

as described above. We also demonstrated that using the balanced highly variable genes yielded comparably accurate estimations (Supplementary Fig. 9).

Estimate gene-wise mean and variance per cell type

Modeling single cell RNA-Seq data is challenging due to cell heterogeneity, technical sensitivity, and noise. Some genes may not be detected by chance, while others may be found to be highly dispersed. These factors can lead to excessive variability even within the same cell type. AdRoit combats high noise and computational complexity by building models with estimated mean and dispersion per gene per cell type. This strategy reduces the data complexity while preserves the cell type specific information.

Although typical analyses of RNA-Seq data start with normalization, AdRoit does not do normalization before the mean estimation. Performing a normalization across all cell types forces every cell type to have the same amount of RNA transcripts, measured by the total unique molecular identifier (UMI) counts per cell. However, different cell types can have dramatically different amounts of transcripts. For example, the amount of RNA transcripts in neuronal cells is about 5 times higher than that in glial cells. Thus, normalization can falsely alter the relative abundance of cell types, misleading the estimation of cell type percentages. To avoid this problem, AdRoit models the means using the raw UMI counts.

Studies have shown that UMI counts follow a negative binomial distribution^{56,57}. We therefore fit negative binomial distributions to genes of each cell type and build the model based on the

estimated means and dispersions from the selected genes. More specifically, let X_{ik} be the set of single cell UMI counts of gene $i \in 1, \dots, I$ for all cells in cell type $k \in 1, \dots, K$. I is the number of selected genes, and K denotes the number of cell types in the single cell count matrix. The distribution of X_{ik} follows a negative binomial distribution,

$$X_{ik} \sim NB(\lambda_{ik}, p_{ik}), \quad (1)$$

where λ_{ik} is the dispersion parameter of the gene i in cell type k , and p_{ik} is the success probability, i.e., the probability of one observed UMI belonging to gene i in cell type k . The two parameters are estimated by MLE. The likelihood function is

$$LH(\lambda_{ik}, p_{ik} | X_{ik}) = \prod_{i=1}^{n_k} f(X_{ik} | \lambda_{ik}, p_{ik}), \quad (2)$$

where n_k is the number of cells in cell type k , and f is the probability mass function of the negative binomial distribution. The MLE estimates are then given by

$$(\hat{\lambda}_{ik}, \hat{p}_{ik}) = \underset{\lambda_{ik}, p_{ik}}{\operatorname{argmax}} LH(\lambda_{ik}, p_{ik} | X_{ik}). \quad (3)$$

Once the success probability and the dispersion are estimated, the mean and the variance of the negative binomial distribution can be computed numerically,

$$\mu_{ik} = \frac{\hat{\lambda}_{ik} \cdot \hat{p}_{ik}}{1 - \hat{p}_{ik}}, \quad (4)$$

$$\sigma_{ik}^2 = \frac{\hat{\lambda}_{ik} \cdot \hat{p}_{ik}}{(1 - \hat{p}_{ik})^2}. \quad (5)$$

Model fitting using MLE has been readily coded in many R packages. We choose the ‘fitdist’ function from the ‘fitdistrplus’ package⁵⁸ for its fast computation speed and flexibility in selecting distributions. The mean and variance estimations are done for each selected gene in each cell type, resulting in a $I \times K$ matrix each.

Cell type specificity of genes

Genes with a cell-type specific expression pattern better distinguish cell types, thus are more important for resolving cell type composition. In line with this property, AdRoit weighs more the genes with a higher cell type specificity. Highly specific genes usually have consistently high expression and thus relatively low variance among cells within a cell type. To compute the cell type specificity of a gene, we first identify the cell type in which the gene has the highest expression (i.e., most expressed cell type), then defines the specificity of this gene as the mean-to-variance ratio within that cell type. A high ratio renders a high weight to the gene in the model. Here the mean and the variance refer to the estimated values from the negative binomial model (μ_{ik} and σ_{ik}^2 in eq. 4 and 5). Let k' be the index of the cell type that has the highest mean expression of gene i ,

$$k' = \underset{k}{\operatorname{argmax}}\{\mu_{ik} \mid k \in 1 \dots K\}, \quad (6)$$

then the weight of the cell type specificity for gene i , denoted w_i^S , is given by,

$$w_i^S = \frac{\mu_{ik'}}{\sigma_{ik'}^2}, \quad (7)$$

and it is computed for each gene in the set of selected genes.

Cross-sample gene variability

The variability of a gene contrasts with how stable a gene is across samples. The idea of weighting genes based on their variability across samples is first explored by Wang et al²², wherein the variability was defined as the cross-sample variance. By weighting down the high variability genes, the authors achieved a great advantage over the traditional unweighted method. Genes with a low cross-sample variability better represent the sample population,

hence are more trustworthy for learning the cell composition. AdRoit incorporates the same notion to weigh the importance of genes, however, it defines the variability more comprehensively. AdRoit acknowledges the dependency between the gene expression level and its variance and thus computes a variance-to-mean ratio (VMR) as the cross-sample variability. Here the mean and the variance are computed across samples. The distribution of the VMR values is less skewed than that of the variance alone. The VMR also circumvents underweighting genes with a low expression, or overweighting genes highly dispersed. Let Y_{ij} denote the counts for gene i in sample $j \in 1, \dots, J$, then

$$Y_{ij} \sim NB(\lambda_{ij}, p_{ij}), \quad (8)$$

where λ_{ij} is the dispersion parameter of the gene i in sample j , and p_{ij} is the success probability. Again, we use MLE to estimate $\widehat{\lambda}_{ij}$ and $\widehat{p}_{ij}$, following which the cross-sample mean and variance can be numerically computed:

$$\mu_i^S = \frac{\widehat{\lambda}_{ij} \widehat{p}_{ij}}{1 - \widehat{p}_{ij}}, \quad (9)$$

$$(\sigma_i^2)^S = \frac{\widehat{\lambda}_{ij} \widehat{p}_{ij}}{(1 - \widehat{p}_{ij})^2}, \quad (10)$$

and the cross-sample variability VMR for gene i is then defined as

$$VMR_i = \frac{(\sigma_i^2)^S}{\mu_i^S} = \frac{1}{w_i^C}, \quad (11)$$

where w_i^C is later used in the model. The cross-sample variability weight is computed for each gene in the set of selected genes.

Typically, the RNA-Seq data of mixed cells to be deconvoluted include multiple samples to control for biological variability. In this case, the VMR is computed as described above using the cross-sample mean and variance of the gene. In case the RNA-Seq data to be deconvoluted lack

multiple samples whereas the single cell reference contains replicates, AdRoit pools all cells per replicate to synthesize multiple compound samples and subsequently estimate the VMRs with them. If neither input has multiple repeats, AdRoit takes a bootstrapping approach to subsample and pool cells from each cell type in the single cell reference several times to generate multiple compound samples.

Gene-wise scaling factor to correct the platform bias

When comparing the RNA-Seq data of cell mixtures to the single cell data, a rescaling factor is often used to account for the library size and platform difference. The existing methods adopt a single rescaling factor for each sample, i.e., all genes in a sample are multiplied by the same factor^{22,23}. This operation is based on a strong assumption that the platform difference impacts every gene equally and linearly scalable among different cell types, which hardly holds. In addition, because linear models are sensitive to outliers, the estimation of cell proportions can be steered away from the truth by genes that are significantly affected by the platform bias. Therefore, applying a uniform scaling factor to all genes is inappropriate.

To overcome this problem, AdRoit estimates a gene-wise scaling factor per sample via an adaptive learning strategy and rescales each gene with its respective scaling factor. To proceed, we first input the mean gene expression from the mixture samples (μ_i^S in eq. 9) and the estimated means of each cell type from the single cell data (μ_{ik} in eq. 4), then apply a non-negative least square regression (NNLS) to get a coarse estimation of the proportions of each cell type, τ_k . For each gene i , a predicted mean expression ($\sum_k^K \widehat{\tau}_k \mu_{ik}$ in eq. 13) in the sample of

mixture cells is computed as the weighted sum of the means of this gene in each cell type

wherein the weights are τ_k . The regression equation is given by,

$$\mu_i^S = A \cdot (\sum_k^K \tau_k \mu_{ik} + \varepsilon), \quad 0 < \tau_k, \sum_k^K \tau_k = 1 \quad (12)$$

where A is a constant to ensure the sum of τ_k 's is 1 and ε is the error term. We use the 'nnls'

function in the 'nnls' package⁵⁹ to estimate τ_k 's. Next, we calculate the ratio between the mean

expression from the mixture samples and the predicted counterpart, and define the gene-wise

rescaling factor as the logarithm of the ratio plus 1,

$$r_i = \log \left(\frac{\mu_i^S}{\sum_k^K \widehat{\tau}_k \mu_{ik}} + 1 \right). \quad (13)$$

Given the dispersed nature of count data, the logarithm of the ratio is a more appropriate

statistic as it results in relatively stable scaling factors. The addition of 1 avoids taking logarithm

on zeros. By multiplying the gene-wise rescaling factor, the genes affected more strongly by the

platform bias (i.e., "outliers") receive a larger correction (Fig. 1b).

Weighted and regularized loss function for training

AdRoit employs a non-negative least square regression model to infer the cell compositions in a

RNA-Seq sample of cell mixtures. The model (eq. 14) approximates the gene expression

observed in the mixture sample by the weighted (the weights are the mixing percentages) sum

of the cell type-specific gene expressions from the single cell reference, after correcting the

platform difference.

$$y_{ij} \approx r_i \cdot \sum_k^K \beta_k \widehat{\mu}_{ik} \quad (\beta_1, \dots, \beta_K > 0) \quad (14)$$

where y_{ij} is the expression of gene i in the mixture transcriptome sample j ; the coefficient β_k is

the mixing proportion for cell type k ; $\widehat{\mu}_{ik}$ is the estimated mean expression of gene i in cell type

k computed according to eq. 4; r_i is the gene-wise scaling factor computed by eq. 13 to correct the bias due to the technology difference. To infer the β_k coefficients, AdRoit uniquely incorporates all the factors discussed in the previous sections. This is implemented in a weighted sum-of-squared loss function L , where the weights consist of two components, w_i^C in eq. 7 and w_i^S in eq. 11. Thus, a larger weight is given to the genes with a higher cell type specificity and a lower cross-sample variability. In cases of complex tissues where many similar cell subtypes exist, strong collinearity among the subtypes can render the model sensitive to noise and prone to overfitting. AdRoit handles this problem by including an L2 norm of the mixing proportions as the regularization component in the loss function. For a compound transcriptome sample j , the loss function is given by,

$$L_j(\beta_1, \dots, \beta_K | y_{ij}, w_i^C, w_i^S, r_i, \widehat{\mu}_{ik}) = \sum_i^I w_i^C \cdot w_i^S \cdot (y_{ij} - r_i \cdot \sum_k^K \beta_k \widehat{\mu}_{ik})^2 + \sum_k^K \beta_k^2. \quad (15)$$

where i is the index of a gene among the selected genes used for the deconvolution; y_{ij} , β_k , r_i , and $\widehat{\mu}_{ik}$ are the same as defined in eq. 14; w_i^S and w_i^C are computed as described above in eq. 7 and 11, respectively. The β_k coefficients are estimated by minimizing the loss function subject to the constraint $\beta_1, \dots, \beta_K > 0$,

$$\widehat{\beta}_1, \dots, \widehat{\beta}_K = \underset{\beta_1, \dots, \beta_K}{\operatorname{argmax}}_{\beta_1, \dots, \beta_K > 0} L_j. \quad (16)$$

The minimization is done by a gradient projection method proposed by Byrd et al⁶⁰. We derive the gradient function by taking the partial derivative of the loss function with respect to β_k ,

$$G_k = \nabla_{\beta_k} L_j = -2 \sum_i^I r_i \cdot \widehat{\mu}_{ik} \cdot w_i^C \cdot w_i^S \cdot (y_{ij} - r_i \cdot \sum_k^K \beta_k \widehat{\mu}_{ik}) + 2\beta_k. \quad (17)$$

AdRoit uses the function ‘optim’ from the R package ‘stats’ to perform the minimization⁶¹, providing the loss function (eq. 15) and the gradient (eq. 17). To get the final estimates of cell type proportions, we rescale the coefficients β_k ’s to ensure a summation of 1,

$$\theta_k = \frac{\widehat{\beta}_k}{\sum_k^K \widehat{\beta}_k}. \quad (18)$$

Each mixture RNA-Seq sample j is independently estimated by the model described above.

Evaluation statistics

We compared the estimated cell type proportions with the ground truth by calculating the following four statistics. The mean absolute difference (mAD) and root mean square deviation (RMSD) are given by,

$$mAD = \frac{\sum_k^K |\theta_k - \theta_k^0|}{K} \quad (19)$$

$$RMSD = \frac{\sum_k^K (\theta_k - \theta_k^0)^2}{K} \quad (20)$$

where θ_k and θ_k^0 are the estimated proportions and true proportions, respectively.

Pearson correlation coefficient is computed as,

$$\rho_p = \frac{\sum_k^K (\theta_k - \overline{\theta_k})(\theta_k^0 - \overline{\theta_k^0})}{\sqrt{\sum_k^K (\theta_k - \overline{\theta_k})^2} \sqrt{\sum_k^K (\theta_k^0 - \overline{\theta_k^0})^2}} \quad (21)$$

where $\overline{\theta_k}$ and $\overline{\theta_k^0}$ are the means of the estimated proportions and true proportions,

respectively. Spearman correlation coefficient is given by,

$$\rho_s = \frac{\sum_k^K (r_k - \overline{r_k})(r_k^0 - \overline{r_k^0})}{\sqrt{\sum_k^K (r_k - \overline{r_k})^2} \sqrt{\sum_k^K (r_k^0 - \overline{r_k^0})^2}} \quad (22)$$

where r_k is the rank of θ_k .

Generation of synthesized bulk RNA-Seq and spatial transcriptomics data

Bulk RNA-Seq data used for the benchmarking were synthesized by adding up the raw UMI counts per gene from all cells within a subject. Let t_k denote a cell of cell type k , $t_k \in 1, \dots, T_k$, where T_k is the total number of cells in cell type k . Let Y_{ij}^B be the count of gene i in a synthesized bulk sample associated with the subject j , and X_{ijt_k} be the UMI count of gene i in cell t_k within the subject j , then

$$Y_{ij}^B = \sum_k^K \sum_{t_k}^{T_k} X_{ijt_k} \quad (23)$$

The true proportion of cell type k is given by,

$$\theta_k^0 = \frac{T_k}{\sum_k^K T_k} \quad (24)$$

To simulate the spatial transcriptomic spots, we first sampled 10 cells without replacement from each cell type, then weighed them by their respective mixing proportions before adding them up. For example, to simulate a spot with p_k percent of cell type k , the count Y_{ij}^S of gene i in a spatial spot j is given by,

$$Y_{ij}^S = \sum_k^K p_k \sum_{n=1}^{10} X_{ikn} \quad (25)$$

where X_{ikn} is the UMI count of gene i in a selected cell n of cell type k . For each mixing scheme, the simulation was repeated 100 times.

Single cell RNA sequencing of mouse dorsal root ganglion

As described previously⁶², lumbar DRGs were isolated from five 18-week old adult C57BL/6 mice (three males and two females) and transferred to a dissociation buffer (Dulbecco's

747 modified Eagle's medium supplemented with 10% heat-inactivated Fetal Calf Serum) (Gibco; cat
748 # A38400-02). To generate a single cell suspension, DRGs were subjected to a 2 step-enzymatic
749 dissociation followed by a mechanical dissociation. In brief, DRGs were first incubated with
750 0.125% collagenase P from Clostridium histolyticum (Roche Applied Science; cat #
751 11249002001) for 90 minutes in an Eppendorf Thermomixer C (37°C; intermittent 750 rpm
752 shaking for about 10 sec every 2 minutes). Then, DRGs were transferred to a Hank's Balanced
753 Salt Solution (HBSS, Mg²⁺ and Ca²⁺ free; Invitrogen) supplemented with 0.25% Trypsin
754 (Worthington biochemical corp.; cat # LSoo3707) and 0.0025% EDTA and incubated for 10
755 minutes at 37°C in the Eppendorf Thermomixer C. Trypsin was neutralized by the addition of 2.5
756 mg/ml MgSO₄ (Sigma; cat #M-3937) and DRGs were triturated with Pasteur pipettes. The
757 resulting cell suspension was passed through a 70 µm mesh filter to remove remaining chunks
758 of tissues and centrifuged for 5 minutes at 2500 rpm at room temperature. The pellet was
759 resuspended in HBSS (Ca²⁺, Mg²⁺ free; Invitrogen) and the cell suspension was run on a 30%
760 Percoll Plus gradient (Sigma GE17-5445-02) to further remove debris. Finally, cells were
761 resuspended in PBS supplemented with 0.04% BSA at a concentration of 200 cells/µl and cell
762 viability was determined using the automated cell analyzer NucleoCounter® NC-250™. The
763 suspended single cells were loaded on a Chromium Single Cell Instrument (10X Genomics) with
764 about 6000 cells per lane to minimize the presence of doublets. 2000-3000 cells per lane were
765 recovered. RNA-Seq libraries were constructed using Chromium Single Cell 3' Library, Gel Beads
766 & Multiplex Kit (10X Genomics). Single end sequencing was performed on Illumina NextSeq500.
767 Read 1 starts with a 26-bp UMI and cell barcode, followed by an 8-bp i7 sample index. Read 2
768 contains a 55-bp transcript read. Sample de-multiplexing, alignment, filtering, and UMI

counting were conducted using Cell Ranger Single-Cell Software Suite⁶³ (10X Genomics, v2.0.0).
Mouse mm10 Genome assembly and UCSC gene model were used for the alignment.

Data preprocessing

DRG single cell data

The UMI data output from the Cell Ranger Single-Cell Software Suite (10X Genomics, v2.0.0) was analyzed using Seurat package⁶⁴ to assess the cell quality and identify cell types, similar to what was described previously⁴². Cells with the number of detected genes less than 500 or over 15000, or with a UMI ratio of mitochondria encoded genes versus all genes over 0.1 were also removed. The UMI data was normalized by the ‘NormalizeData’ method in Seurat with default settings. To avoid potential sample-to-sample variation caused by the technical variation at various experiment steps, we employed Seurat data integration method. The top 2000 variable genes of each of the 5 DRG samples were identified using ‘FindVariableFeatures’ with selection.method=‘vst’. Based on the union of these variable genes, the anchor cells in each sample were identified by ‘FindIntegrationAnchors’. All the samples were then integrated by ‘IntegrateData’. We subsequently scaled the integrated data (‘ScaleData’) and performed dimension reduction (‘RunPCA’). Cells were then clustered based on the first 15 principal components by applying ‘FindNeighbors’ and ‘FindClusters’ (resolution=0.6, algorithm=1). Marker genes for each cluster were identified using ‘FindAllMarkers’. Parameters were used such that these genes were expressed in at least 25% of the cells in the cluster, and on average 2-fold higher than the rest of cells with a multiple-testing adjusted Wilcoxon test p value of less than 0.01. The specificity of the canonical cell type-specific genes or cell cluster-specific genes

were further examined by visualizations (Supplementary Fig. 4) and used to define the cell type for each cluster. At the end, the original UMI data from 17271 genes and 3352 cells that passed the quality control were organized into a matrix (genes as rows and cell identifiers as columns). This matrix, together with the cell type label for each cell therein, were loaded into AdRoit as the reference profiles.

Mouse brain single cell atlas data

The scRNA-Seq reference data of the mouse brain were obtained from Zeisel et. al³². Among all the available data, we only retained 96,572 cells that were acquired from the brain regions, had an assigned cell type by the authors and a minimal total UMI of 1000. These cells corresponded to 183 clusters at the finest taxonomy level in the original study. As many of the clusters are highly similar, we decided to merge some of them to simplify the reference landscape. First, the top 50 cluster enriched markers were derived using Scanpy⁶⁵ via the 'rank_genes_groups' function (method='wilcoxon'), following the normalization ('normalize_per_cell'), log transformation ('log1p') and regressing out ('regress_out') the variances associated with the total UMI and the percentage of mitochondrial chromosome encoded genes per cell. Then, the pair-wise overlapping p-values among the clusters were calculated using the top 50 marker genes assuming a hypergeometric null distribution. Last, clusters with overlapping p-values more significant than 1e-10 were merged and new names were assigned by combinedly considering the original annotation, the molecular features and the specificity to certain brain regions. A total of 46 cell types were determined that cover all the 12 brain regions and their important substructures⁴⁰ (Supplementary Table 7). To make the reference dataset more

manageable in size and more balanced in the representation of cell types, we down sampled each cluster to no more than 360 cells. A final set of 14,666 cells over 46 cell types was used for the deconvolution of the mouse brain spatial transcriptome data.

Human Islets single cell and bulk RNA-Seq data

We used 1492 high quality human islets single cells and the associated annotations from Xin et al⁴¹. The RPKM expression table was directly downloaded and used as-is. The RNA-FISH data was also from this study⁴¹. The real bulk RNA-Seq data of human islets was acquired from a large scale study referred in multiple publications^{41,43,44}. We only included the data from donors with a valid HbA1C level measurement in the regression analysis of the Beta cell proportion with respect to the HbA1C level (Fig. 5c, Supplementary Table 13).

Trabecular Meshwork single cell data

We acquired the UMI counts, tSNE coordinates and the cell type annotation of 8759 high quality cells from the authors of Patel et al⁴². Details of the quality control and the cell type identification were presented in the original publication.

Mouse Brain Spatial transcriptomics data by 10x Visium platform

The filtered count matrix, tissue image and the spatial coordinates of a coronal section of an adult C57BL/6 mouse brain were download from the 10x Genomics website (see Data availability). A total of 2698 in-tissue spots were provided in the data set and used as-is.

835 *Mouse Brian ISH images*

836 The ISH images were directly downloaded from Allen mouse Brain Atlas⁴⁰ by searching the gene
837 names. The images were used without further editing except for cropping.

838

839 **Data availability**

840 The mouse DRG single cell data were deposited at NCBI GEO (accession number: GSE163252).

841 The bulk RNA-Seq and RNA-FISH data for human pancreatic islets were initially published as

842 aggregated data where the data processing and experimental procedure were described

843 therein^{41,43,44}. We acquired the individual sample data from the authors and released them

844 along with the current study (Supplementary Table 10 and Supplementary Table 12). The other

845 public data analyzed in this study were obtained using GEO accession number GSE81608

846 (human pancreatic islets single cell data), NCBI SRA accession number PRJNA616025 (human

847 trabecular meshwork single cell data), and NCBI SRA accession number SRP135960 (mouse

848 brain single cell data). The 10x Genomics PBMC data was downloaded from the link

849 (<https://support.10xgenomics.com/single-cell-gene-expression/datasets/1.1.0/pbmc3k>), and

850 the Visium mouse brain spatial transcriptomic data was downloaded from the link

851 ([https://support.10xgenomics.com/spatial-gene-](https://support.10xgenomics.com/spatial-gene-expression/datasets/1.1.0/V1_Adult_Mouse_Brain_Coronal_Section)

852 [expression/datasets/1.1.0/V1_Adult_Mouse_Brain_Coronal_Section](https://support.10xgenomics.com/spatial-gene-expression/datasets/1.1.0/V1_Adult_Mouse_Brain_Coronal_Section)).

853

854 **Code availability**

855 AdRoit's source code is available on Github (<https://github.com/TaoYang-dev/AdRoit>).

856

Software

The statistical analyses were done with R statistical software (v3.6.0)⁶¹ and python (v3.7.2)⁶⁶. The packages used include Seurat (v3.0.1)⁶⁴, scanpy (v1.6.0)⁶⁵, dplyr (v0.8.0.1)⁶⁷, doParallel (v1.0.14)⁶⁸, data.table (v1.12.4)⁶⁹, fitdistrplus (v1.1-1)⁵⁸, nnls (v1.4)⁵⁹, MuSiC (v0.1.1)²², BisqueRNA (v1.0.4)²¹, SPOTlight (v1.0.4)³⁸, Cell2location (v0.05-alpha)³⁹, Stereoscope(v_03)²³.

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1022 **Acknowledgements**

1023 We thank Yurong Xin for pointing us to the relevant public data resource. We also thank Gabor
1024 Halasz and Yuan Zhu for the helpful discussions regarding the memory usage, and Shawn
1025 Mishra for the manuscript proofreading.

1026

1027 **Author contributions**

1028 Y.B., T.Y., N.A.-H., M.L.-F., L.E.M. and G.S.A. designed the research. T.Y., Y.B., and W.F.
1029 developed the algorithm. T.Y., Y.B. and J.K. participated in the data analysis. M.S. and R.B.
1030 performed the DRG tissue collection. C.A. performed the single cell library preparation and
1031 sequencing experiment. Y.B., T.Y. and N.A.-H. wrote the manuscript.

1032

1033 **Competing interests**

1034 T.Y., Y.B., W.F. and G.S.A. have filed a patent application relating to the AdRoit computational
1035 framework. M.L.-F. is an employee of Cellular Longevity. All other authors are employees
1036 and/or shareholders of Regeneron Pharmaceuticals, although the manuscript's subject matter
1037 does not have any relationship to any products or services of this corporation.

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1039 **Figure legends**

1040 **Fig. 1: Schematic representation of AdRoit computational framework. a,** AdRoit inputs
1041 compound (bulk or spatial) RNA-Seq data, single cell RNA-Seq data and cell type annotations. It
1042 first selects informative genes and estimates their means and dispersions, then computes the
1043 cell type specificity of genes. Depending on the availability of multiple samples, cross-sample
1044 gene variability is derived from either the compound RNA-Seq, or the single cell data (dashed
1045 arrow; see also Methods). Lastly the gene-wise scaling factors are computed to correct the
1046 platform bias between the compound and the single cell RNA-Seq data. These quantities are
1047 used in a weighted regularized model to infer the cell type composition. **b,** A mock example to
1048 illustrate the role of the gene-wise scaling factor. Ideally, an accurate estimation of the cell
1049 proportions should be represented by the slope of the green line, however fitting in the
1050 presence of outlier genes would result in the red line. Outlier genes exist because the platform
1051 bias affects genes differently. AdRoit adopts an adaptive learning approach that first learns a
1052 coarse estimation of the slope (red line), from which the gene-wise scaling factors are derived
1053 and applied to the outlier genes, moving them toward the green line. The more deviated the

gene, the larger the correction (i.e., longer arrows). After the adjustment, the new estimated slope (blue line) is closer to the truth (green line), thus is a more accurate estimation.

Fig.2: Benchmark on simulated bulk data generated from the trabecular meshwork (TM) single cells. **a**, AdRoit has the closest estimation to the true cell proportion comparing to Bisque, MuSiC and SPOTlight. Each dot is a cell type from a donor. **b**, For each cell type in TM, AdRoit has the smallest differences from the true cell type proportion and the smallest variance of estimates across the 8 donors. For each cell type, a dot on the graph denotes a donor, and the bars represent the $1.5 \times$ interquartile ranges. The reference used for deconvoluting each synthetic bulk sample excludes the cells used to synthesize that sample (leave-one-out).

Fig. 3: AdRoit can achieve a high granularity and exhibits high sensitivity and specificity in complex tissues. **a**. AdRoit is accurate in deconvoluting the simulated bulk samples that contain a mixture of similar cell types from myeloid or lymphoid lineage. The vertical dashed lines indicate the true mixing proportions. CD14⁺ monocytes, FCGR3A⁺ monocytes and dendritic cells (DC) were mixed under three schemes of proportions: 0.33:0.33:0.33 (mix0), 0.1:0.45:0.45 (mix1) and 0.1:0.3:0.6 (mix2). The same ratios were applied to the mixtures of naïve CD4⁺ T, memory CD4⁺ T and CD8⁺ cells. **b**, AdRoit's estimates are more accurate and specific than those from Bisque, MuSiC and SPOTlight on synthetic samples that contain only 6 out of the 12 cell types. The deconvolution was done using all 12 cell types as the reference. A pair of size-matched blue (true value) and red (estimated value) bubbles indicate an accurate prediction. Red-only and blue-only bubbles mark false positives and false negatives, respectively. **d**,

Receiver operating characteristic (ROC) curve shows AdRoit has a significantly higher area under the curve (AUC) than other methods, meaning better sensitivity and specificity. **e**, Scatterplots between the ground truth and the deconvoluted cell proportions in the simulated bulk samples of high complexity (mixtures of 30 cell types). **f**, ROC curves show AdRoit has the best AUC among all methods on highly complex cell constitutions.

Fig. 4: Benchmark on scRNA-Seq data from dorsal root ganglion (DRG) where many closely related subtypes of neuronal cells exist. **a**, 14 cell types are identified from scRNA-Seq samples of 5 mice, including multiple subtypes of neurofilaments (NF), peptidergic (PEP), and non-peptidergic (NP) neurons. **b**, Benchmarking with the synthetic data shows the cell type proportions inferred by AdRoit are highly accurate. In particular, AdRoit remains a high accuracy when the cells are rare (e.g., < 5%; see also the zoom-in inserts). Each dot represents a cell type from one sample. **c**, For each sample, mAD, RMSD, Pearson and Spearman correlations are compared across four methods. AdRoit has the lowest mAD and RMSD, and the highest Pearson and Spearman correlations. In addition, AdRoit's estimation is the most stable across samples. Each dot on the boxplot is a sample. Same samples are chained by the dotted lines across the metrics. The deconvolution was done by using the leave-one-out strategy.

Fig. 5: AdRoit is more accurate and sensitive on spatial spots simulated from real DRG cells. **a**, Estimations from AdRoit, Cell2location, Stereoscope and SPOTlight on simulated spatial spots that contain 5 PEP neuron subtypes. True mixing proportions are denoted by the red dashed lines. Three schemes are presented: 1) the proportions of 5 PEP cell types are the same and

1098 equal to 0.2; 2) PEP1_Dcn is 0.1 and the other 4 are 0.225; 3) PEP1_Dcn and
 1099 PEP1_S100a11.Tagln2 are 0.1, PEP1_Slc7a3.Sstr2 and PEP2_Htr3a.Sema5a 0.2 are 0.2, and
 1100 PEP3_Trpm8 is 0.4. **b**, The performance of AdRoit, Cell2location, Stereoscope, and SPOTlight in
 1101 estimating rare cell populations in the spatial spots. The spots contain a mixture of three PEP
 1102 cell subtypes (i.e., PEP1_Slc7a3.Sstr2, PEP2_Htr3a.Sema5a and PEP3_Trpm8), with the percent
 1103 of PEP3_Trpm8 ranging from 1% to 10% and the other two cell types sharing the remaining
 1104 proportion equally. **c**, Compare the rate of detecting rare cells in simulated spots. An inferred
 1105 percent greater than 0.5% is deemed as a positive detection. Six sets of cell mixtures are
 1106 employed: NF_Calb1 with NF_Pvalb and NF2_Ntrk2.Necab2 (NP subtypes), NP_Mrgpra3 with
 1107 NP_Mrgprd and NP_Nts (NP subtypes), PEP3_Trpm8 with PEP1_Slc7a3.Sstr2 and
 1108 PEP2_Htr3a.Sema5a (PEP subtypes), NF_Calb1 with Th, satellite glia and endothelial (NF_Calb1
 1109 + others), NP_Mrgpra3 with Th, satellite glia and endothelial (NP_Mrgpra3 + others), and
 1110 PEP_Trpm8 with Th, satellite glia and endothelial (PEP_Trpm8 + others). In each set, the first
 1111 cell type listed is the target of detection and varies its percent from 1% to 10%. The rest cell
 1112 types split the remaining proportion evenly. The red dashed lines mark the detection rate of
 1113 90%.

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1115 **Fig. 6: Applications to real-world bulk RNA-Seq data and mouse brain spatial transcriptome**
 1116 **data. a**, The deconvoluted cell compositions in the real bulk RNA-Seq data of human Islets are
 1117 highly reproducible for the repeated samples from the same donor. **b**, AdRoit estimation of the
 1118 cell type proportions agrees with the RNA-FISH measurements. **c**, AdRoit-inferred Beta cell
 1119 proportions in type 2 diabetes patients are significantly lower than those in healthy subjects. In

1120 addition, the estimated proportions have a significant negative linear association with the
 1121 donor HbA1C levels. **d**, The spatial mapping of four mouse brain cell types is consistent with the
 1122 locations of four region-specific markers shown on the ISH images obtained from Allen mouse
 1123 brain atlas⁴⁰. The four genes, Spink8, C1ql2, Clic6 and Synpo2, were identified by Zeisel et al³² as
 1124 markers of the hippocampal field CA1, dentate gyrus, choroid plexus and thalamus,
 1125 respectively.

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