1   Reference-free cell-type deconvolution of pixel-resolution spatially resolved transcriptomics data

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20

21 **Abstract**

22 Recent technological advancements have enabled spatially resolved transcriptomic profiling but

23 at multi-cellular pixel resolution, thereby hindering the identification of cell-type spatial co-

24 localization patterns. We developed STdeconvolve as an unsupervised approach to deconvolve

25 underlying cell-types comprising such multi-cellular pixel resolution spatially resolved

26 transcriptomics datasets. We show that STdeconvolve effectively recovers the putative

27 transcriptomic profiles of cell-types and their proportional representation within spatially

28 resolved pixels without reliance on external single-cell transcriptomics references.

29

30 **Main**

31   Delineating the spatial organization of transcriptionally distinct cell-types within tissues

32 is critical for understanding the cellular basis of tissue function[1]. Recent technologies have

33 enabled spatially resolved transcriptome (ST) profiling within tissues at multi-cellular pixel-

34 resolution[2]. As such, these ST measurements represent cell mixtures that may comprise multiple

35 cell-types. This lack of single-cell resolution hinders the characterization of cell-type specific

36 spatial organization. To address this challenge, supervised deconvolution approaches such as

37 SPOTlight[3] and RCTD[4] have recently been developed to predict the proportion of cell-types

38 within ST pixels. However, these supervised deconvolution approaches rely on the availability of

39 a suitable single-cell reference, which may present limitations if such a reference does not exist

40 due to budgetary, technical[5], or biological limitations[6]. Here, we developed STdeconvolve

41 (available at https://github.com/JEFworks-Lab/STdeconvolve and as Supplementary Software)

42 as an unsupervised, reference-free approach for deconvolving multi-cellular pixel resolution ST

43 data (Figure 1). Given a counts matrix of ST data, STdeconvolve infers the putative

44   transcriptomic profiles of distinct cell-types and their proportional representation within each

45   multi-cellular spatially resolved ST pixel (Methods). Briefly, STdeconvolve first feature selects

46   for genes likely to be informative of transcriptionally distinct cell-types. STdeconvolve then

47   builds on Latent Dirichlet Allocation (LDA)[7] to estimate the number of transcriptionally distinct

48   cell-types, K, and deconvolves these K cell-types across ST pixels. STdeconvolve leverages

49   several unique features of ST data that make this application of LDA particularly amenable

50   (Supplementary Note 1, 2).

51   As a proof of concept, we first evaluated the performance of STdeconvolve in recovering

52   the proportional representations of cell-types and their transcriptomic profiles using simulated

53   ST data. This was done by aggregating single-cell resolution multiplex error-robust fluorescence

54   *in situ* hybridization (MERFISH) data of the mouse medial pre-optic area (MPOA)[8] into 100

55   μm^2 pixels (Figure 2A, Supplementary Figure S1A-B, S2, Methods). Applying STdeconvolve,

56   we identified K=9 transcriptionally distinct cell-types and deconvolved their transcriptomic

57   profiles and proportional representation in each simulated pixel (Figure 2B, Supplementary

58   Figure S1C, S3A). To infer the identities of the deconvolved cell-types for benchmarking

59   purposes, we matched their deconvolved transcriptional profiles with the transcriptional profiles

60   of ground truth cell-types (Methods) (Supplementary Figure S3B-C). We observed strong

61   correlations between the transcriptomic profiles of each deconvolved cell-type and matched

62   ground truth cell-type across genes (Figure 2C) and, likewise, between the proportions of each

63   deconvolved cell-type and matched ground truth cell-type across simulated pixels (Figure 2D).

64   Some deconvolved cell-types such as cell-types X2 and X8 both matched to excitatory neurons

65   while cell-types X4 and X7 both matched to inhibitory neurons. Further partitioning the ground-

66   truth excitatory and inhibitory cell-types into additional sub-types (76 total) based on previous

67   annotations[8] found that these deconvolved cell-types correlated with specific combinations of

68   neuronal sub-types (Supplementary Figure S3D). When we further expanded the number of

69   deconvolved cell-types to $K=76$, we were able to identify deconvolved cell-types that are highly

70   correlated in terms of both transcriptional profiles and pixel proportions to finer neuronal

71   subtypes as well as rare cell-types such as pericytes and microglia (Supplementary Figure 3E-F,

72   Supplementary Note 2, 3). In addition, as current ST technologies allow for spatial

73   transcriptomic profiling at varying resolutions[2], we further simulated another ST dataset at 20

74   μm^2 resolution and observed similarly strong correlations between the deconvolved cell-type

75   transcriptomic profiles and proportions with the ground truth by STdeconvolve (Supplementary

76   Figure S4).

77   Using our simulated 100 μm^2 resolution ST data of the MPOA, we also compared

78   STdeconvolve to existing supervised deconvolution approaches SPOTlight and RCTD. For an

79   ideal single cell transcriptomics reference, we used the original single-cell MERFISH data. We

80   evaluated the performance of each approach using the root-mean-square-error (RMSE) of the

81   deconvolved cell-type proportions compared to ground truth across simulated pixels (Methods).

82   In general, we find the performance of STdeconvolve to be comparable to SPOTlight and RCTD

83   (Supplementary Fig. S5A). One potential limitation of such existing supervised deconvolution

84   approaches is their reliance on a suitable single-cell reference. We thus sought to evaluate their

85   performance when a suitable single-cell reference is not available by removing neuronal cell-

86   types from the MERFISH single-cell reference. Reevaluating performance resulted in an increase

87   in RMSE for both SPOTlight and RCTD (Supplementary Fig. S5B).

88   We next evaluated the performance of STdeconvolve by analyzing 100 μm^2 resolution

89   ST data of the mouse main olfactory bulb (MOB)[9]. The MOB consists of multiple bilaterally

90   symmetric and transcriptionally distinct cell layers due to topographically organized sensory

91   inputs[10]. While previous clustering analysis of MOB ST data revealed coarse spatial organization

92   of coarse cell layers, finer structures such as the rostral migratory stream (RMS) could not be

93   readily observed (Supplementary Figure S6A-B). We applied STdeconvolve to identify K=12

94   transcriptionally distinct cell-types (Figure 2E, Supplementary Figure S6C) that either

95   overlapped with or further split coarse cell layers previously identified from clustering analysis

96   (Supplementary Fig S6D). In particular, while deconvolved cell-type X7 overlapped with the

97   granule cell layer previously identified from clustering analysis, it was spatially placed where the

98   RMS is expected[11] (Figure 2F). Upregulated genes in its deconvolved transcriptional profile,

99   including *Nrep*, *Sox11,* and *Dcx*, are known to be associated with neuronal differentiation or

100   upregulated in neuronal precursor cells within the RMS[12] and mark the expected spatial

101   organization based on ISH staining[13] (Figure 2G, Supplementary Figure S6E). This suggests that

102   deconvolved cell-type X7 corresponds to the neuronal precursor cell-type within the RMS

103   unidentified from clustering analysis. Again, we compared STdeconvolve to SPOTlight and

104   RCTD using an appropriate MOB scRNA-seq reference[14] and found a high degree of

105   correspondence among all evaluated methods (Supplementary Figure S7).

106   We again compared the performance of such supervised deconvolution approaches when

107   a suitable single-cell reference is lacking by removing olfactory ensheathing cells (OECs) from

108   the MOB scRNA-seq reference. OECs were initially predicted to be enriched in the olfactory

109   nerve layer by all evaluated methods (Supplementary Fig S8A-C). However, given this new

110   reference without OECs, SPOTlight and RCTD erroneously predicted N2 cells to be enriched in

111   the olfactory nerve layer and highly abundant (Supplementary Figure S8A, S8D) even though N2

112   cells were initially predicted to be rare by all methods. In addition, we trained SPOTlight and

113   RCTD on a scRNA-seq reference from the mouse cortex[13] resulting in the vascular

114   leptomeningeal (VLMC) cell cluster of the cortex reference to be erroneously predicted as highly

115   enriched in the olfactory nerve layer (Supplementary Figure S9). As such, supervised

116   deconvolution approaches may be sensitive to the single-cell transcriptomics reference used.

117   Finally, to demonstrate the potential of an unsupervised, reference-free approach, we

118   applied STdeconvolve to 100 μm^2 resolution ST data of 4 breast cancer sections[15]. Here, a

119   matched scRNA-seq reference was not available and using a scRNA-seq reference from another

120   breast cancer sample may be inappropriate due to potential inter-tumoral heterogeneity[16].

121   Transcriptional clustering of the ST pixels previously identified 3 transcriptionally distinct

122   clusters that corresponded to 3 histological regions of the tissue: ductal carcinoma *in situ*,

123   invasive carcinoma, and non-malignant[15] (Supplementary Figure S10A-B). However, the tumor

124   microenvironment is a complex milieu of many additional cell-types[17]. We applied

125   STdeconvolve to identify additional cell-types and interrogate their spatial organization,

126   resulting in *K*=15 identified cell-types (Figure 3A, Supplementary Figure S10C). Of these,

127   deconvolved cell-types X3, X13, and X15 corresponded spatially and had deconvolved

128   transcriptional profiles consistent with the non-malignant, ductal in situ carcinoma, and invasive

129   carcinoma annotations, respectively (Supplementary Figure S10D, S11). However, deconvolved

130   cell-type X15 was spatially enriched at the interface of the cancerous and non-malignant regions

131   of the tissue (Figure 3B). Highly expressed genes for deconvolved cell-type X15 included

132   immune genes such as *CD74* and *CXCL10* and gene set enrichment analysis suggested

133   significant enrichment in immune processes (Figure 3C-E, Supplementary Figure S12,

134   Supplementary Table S1), suggesting that deconvolved cell-type 15 may correspond to immune

135   cells. Such spatial organization of immune cells along a tumor boundary has been previously

6

136   implicated to play a role in breast cancer prognosis[18]. Therefore, STdeconvolve may be able to

137   assist in deconvolving transcriptionally distinct cell-types in heterogeneous tissues to discover

138   potentially clinically relevant spatial organization.

139   In conclusion, we have developed STdeconvolve as a tool for analyzing ST data to

140   recover cell-type proportion and transcriptional profiles without reliance on single-cell

141   transcriptomics references. We show that STdeconvolve can recapitulate expected biology and

142   provide competitive performance to existing supervised methods when suitable single-cell

143   references are available, as well as potentially superior performance when suitable single-cell

144   references are not available. In general, we anticipate that STdeconvolve will help interrogate the

145   spatial relationships between transcriptionally distinct cell-types in complex heterogeneous

146   tissues.

147

148   **Methods**

149

150   **STdeconvolve Overview**

151   STdeconvolve uses latent Dirichlet allocation (LDA)[7], a generative probabilistic model, to

152   deconvolve the latent cell-types contained within multi-cellular pixels of spatially resolved

153   transcriptome (ST) measurements. In this context, each pixel is defined as a mixture of K cell-

154   types represented as a multinomial distribution of cell-type probabilities (θ), and each cell-type

155   is defined as a probability distribution over the genes (β) present in the ST dataset.

156

157   *LDA Modeling*

7

158   The ST dataset is represented as a $D \times V$ matrix of discrete gene counts for each pixel d and

159   gene v. The number of total gene counts in a given pixel d is N_d.

160

161   As a generative probabilistic model, the LDA model generates a set of new pixels as follows:

162

163   For each pixel d:

164   a. draw a cell-type distribution $\theta_d \sim Dir(\alpha)$, where θ_d is a multinomial distribution of

165   length K drawn from a uniform Dirichlet distribution with scaling parameter α.

166   b. for each gene count n in N_d:

167   i.   draw cell-type $k_{d,n} \sim mult(\theta_d)$

168   ii.   draw gene $v_{d,n} \sim mult(\beta_{k_{d,n}})$

169

170   The central goal is to identify the posterior distribution of the latent parameters given the input

171   data, where for each pixel d:

172
$$p(\theta_d, \boldsymbol{k} \mid \boldsymbol{v}, \alpha, \beta) = \frac{p(\theta_d, \boldsymbol{k}, \boldsymbol{v} \mid \alpha, \beta)}{p(\boldsymbol{v} \mid \alpha, \beta)}$$

173   where $\boldsymbol{k}$ is a vector of N_d cell-types associated with each gene in pixel d, and $\boldsymbol{v}$ is the vector of

174   N_d genes for pixel d. A variational expectation-maximization approach is used to estimate the

175   values of the latent parameters[7,19]. By default, β is initialized with 0 for all cell-types and genes,

176   and α as 50/K.

177

178   The resulting estimated θ and β matrices represent the deconvolved proportions of cell-types in

179   each pixel and the gene expression profiles for each cell-type, scaled to a library size of 1. β

8

180 represents a $K \times V$ gene-probability matrix for each cell-type k and each gene v with each row

181 summing to 1. The β matrix can be multiplied by a scaling factor of one million to be more like

182 conventional counts-per-million expression values for interpretability. θ represents a $D \times K$

183 pixel-cell-type proportion matrix for each pixel d and each cell-type k. LDA modeling in

184 STdeconvolve is implemented through the `topicmodels` R package[19].

185

186 Of note, LDA inherently assumes for each pixel, there are a few cell-types present with high

187 probability. We find this assumption reasonable for ST data due to the limited number of cells

188 being captured within an ST pixel (Supplementary Fig. 1). Likewise, LDA assumes for each cell-

189 type, there is a set of genes associated with high probability. Therefore, STdeconvolve uses

190 feature selection for genes more likely to be associated with cell-types, which can improve cell-

191 type deconvolution.

192

193 *Selection of genes for LDA model*

194 To filter for genes that are more likely to be specifically expressed in particular cell-types to

195 improve cell-type deconvolution by LDA, STdeconvolve first removes genes that are not

196 detected in a sufficient number of pixels. By default, genes detected in less than 5% of pixels are

197 removed. Because LDA attempts to identify tightly occurring, and ideally non-overlapping

198 clusters of genes in the pixels, the most expressed genes in the dataset, as well as genes that are

199 expressed in many pixels may also be removed. By default, genes detected in 100% of pixels are

200 removed. STdeconvolve then selects for significantly overdispersed genes, or genes with higher-

201 than-expected expression variance across pixels, as a means to detect transcriptionally distinct

202 cell-types[20]. We assume that the proportion of cell-types will vary across pixels and thus

203 differences in their cell-type-specific transcriptional profiles manifest as overdispersed genes

204 across pixels in the dataset. Additional gene filtering or cell-type specific marker genes to

205 include in the input ST dataset may also be augmented by the user.

206

207 *Selection of LDA model with optimal number of cell-types*

208 The number of cell-types K in the LDA model must be chosen *a-priori*. To determine the

209 optimal number of cell-types K to set for an LDA model for a given dataset, we fit a set of LDA

210 models using a different value for K over a user defined range of positive integers greater than 1.

211 Additionally, users may select a held-out subset of pixels to then apply the fitted model to. We

212 then compute the perplexity of each fitted model:

213
$$Perplexity(D) = \exp\left\{-\frac{\log(p(D))}{\sum_{d=1}^{D}\sum_{v=1}^{V}n^{(dv)}}\right\}$$

214 Where $p(D)$ is the likelihood of the dataset and $n^{(dv)}$ is the gene count of gene v in pixel d. We

215 can interpret $p(D)$ as the posterior likelihood of the dataset conditional on the cell-type

216 assignments using the final estimated θ and β. The lower the perplexity, the better the model

217 represents the real dataset. Thus, the trend between choice of K and the respective model

218 perplexity can then serve as a guide. By default, the perplexity is computed by comparing $p(D)$

219 to the entire input dataset used to estimate θ and β.

220   In addition, STdeconvolve also reports the trend between K and the number of

221 deconvolved cell-types with mean pixel proportions < 5% (as default). We chose this default

222 threshold based on the difficulty of STdeconvolve, SPOTlight, and RCTD to deconvolve cell-

223 types at low proportions, (i.e., "rare" cell-types) (Supplementary Note 2). We note that as K is

224 increased for fitted STdeconvolve models, the number of such "rare" cell-types generally

225 increases. Such rare deconvolved cell-types are often distinguished by fewer distinct

10

226   transcriptional patterns in the data and may represent non-relevant or spurious subdivisions of

227   primary cell-types. We can use this metric to help set an upper bound on K.

228   Generally, perplexity decreases and the number of "rare" deconvolved cell-types

229   increases as K increases. Given these model perplexities and number of "rare" deconvolved cell-

230   types for each tested K, the optimal K can then be determined by choosing the maximum K with

231   the lowest perplexity while minimizing number of "rare" deconvolved cell-types. To further

232   guide the choice of K, an inflection point ("knee") is derived from the maximum second

233   derivative of the plotted K versus perplexity plot and K versus number of "rare" deconvolved

234   cell-types. We find that the optimal K is stable for models fitted to similar datasets, and that the

235   deconvolved cell-types are highly similar in terms of their deconvolved transcriptional profiles

236   (Supplementary Note 4). Ultimately, the choice of K is left up to the user and can be chosen

237   taking into consideration prior knowledge of the biological system.

238

239   **Simulating ST data from single-cell resolution spatially resolved MERFISH data**

240   MERFISH data of the mouse medial preoptic area (MPOA) was obtained from the original

241   publication[8]. Normalized gene expression values were converted back to counts by dividing by

242   1000 and multiplying by each cell's absolute volume. Datasets for an untreated female animal

243   (FN7, datasets 171021_FN7_2_M22_M26 and 171023_FN7_1_M22_M26) containing counts

244   for 135 genes assayed by MERFISH were used. Genes with non-count expression intensities

245   assayed by sequential FISH were omitted. Counts of blank control measurements were also

246   removed. Cells were previously annotated as being one of 9 major cell-types (astrocyte,

247   endothelial, microglia, immature or mature oligodendrocyte, ependymal, pericyte, inhibitory

248   neuron, excitatory neuron). Cells originally annotated as "ambiguous" were removed from the

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249 dataset to ensure the ground truth was composed of cells with known cell-types. Because certain

250 cell-types are only present in specific regions of the MPOA, we combined 12 tissue sections

251 across the anterior and posterior regions to ensure that all expected cell-types would be present in

252 the final simulated ST dataset. After filtering, the final dataset contained 59651 cells representing

253 9 total cell-types and counts for the 135 genes.

254   To simulate a multi-cellular pixel resolution ST dataset from such single-cell resolution

255 spatially resolved MERFISH data, we generated a grid of squares, each square with an area of

256 100 μm^2. Each square was considered a simulated pixel and the gene counts of cells whose x-y

257 centroid was located within the coordinates of a square pixel were summed together. A grid of

258 square pixels was generated for each of the 12 tissue sections separately and the simulated pixels

259 for all 12 tissue sections were subsequently combined into a single ST dataset. For a given tissue

260 section, the bottom edge of the grid was the lowest y-coordinate of the cell centroids and the left

261 edge of the grid was the lowest x-coordinate. Square boundaries were then drawn from each of

262 these edges in 100 μm^2 increments until the position of the farthest increment from the origin

263 was greater than the highest respective cell centroid coordinate. After generating the grid, square

264 pixels whose edges formed one of the outside edges of the grid were discarded in order to

265 remove simulated pixels, which by virtue of their placement, encompassed space outside of the

266 actual tissue sample. The retained pixels covered 49142 out of the original 59651 cells in the 12

267 tissue sections. This resulted in a simulated ST dataset with 3072 pixels by 135 genes. We used

268 the original cell-type labels of each cell to compute the ground truth proportions in each

269 simulated pixel. Likewise, to generate the ground truth transcriptional profiles of each cell-type,

270 we averaged the gene counts for cells of the same cell-type from the original 59651 cells and

271 normalized the resulting gene count matrix to sum to 1 for each cell-type. To simulate pixels of

272   20 μm², an identical approach was taken using the same cells except that square boundaries were

273   drawn from each edge in 20 μm² increments.

274

275   **Deconvolution of simulated MERFISH ST data**

276   STdeconvolve was applied to the simulated MERFISH MPOA ST dataset. We selected the

277   model with the K with the lowest perplexity and where the number of "rare" cell-types = 0

278   resulting in $K = 9$ detected cell-types. To compare deconvolved cell-types to the ground truth

279   cell-types in the simulated ST dataset, we computed the Pearson's correlation between every

280   combination of deconvolved cell-type and ground truth cell-type transcriptional profile.

281   Likewise, the Pearson's correlation between the pixel proportions of each deconvolved cell-type

282   and ground truth cell-type was computed. After assignment of deconvolved to ground truth cell-

283   types, the ranking of each gene based on its expression level in the transcriptional profile of the

284   deconvolved or ground truth cell-type for each assigned match was compared.

285

286   *Annotation and matching of deconvolved and ground truth cell-types*

287   Each deconvolved cell-type was first matched with the ground truth cell-type that had the highest

288   Pearson's correlation between their transcriptional profiles. This was done by computing the

289   Pearson's correlation between every combination of deconvolved and ground truth cell-type

290   transcriptional profile.

291   The assignment of deconvolved cell-types to ground truth cell-types was confirmed by

292   testing for enrichment of differentially upregulated genes of the ground truth cell-types in the

293   deconvolved cell-type transcriptional profiles. To determine the differentially upregulated genes

294   of the ground truth cell-types, ground truth transcriptional profiles were converted to counts per

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295   thousand and low expressed genes, defined as those with average expression values less than 5,

296   were removed. For each ground truth cell-type, the $\log_2$ fold-change of each remaining gene with

297   respect to the average expression across the other ground-truth cell-types was computed.

298   Differentially upregulated genes were those with $\log_2$ fold-change > 1. We performed rank-based

299   gene set enrichment analysis of the ground truth upregulated gene sets in each deconvolved cell-

300   type transcriptional profile using the `liger` R package[21]. A match to a ground truth cell-type was

301   confirmed and assigned if the ground truth gene set had the lowest gene set enrichment adjusted

302   p-value that was at least < 0.05, followed by the highest positive enrichment score to break ties.

303

304   *Comparison to SPOTlight and RCTD*

305   For both SPOTlight and RCTD, a single cell transcriptomic profile reference was

306   required. To construct this reference, the matrix of gene counts for the 49142 individual cells

307   included in the simulated ST dataset and their predefined cell-type labels were input into the

308   `seurat` R package[22] (v4.0.1) as recommended in both the SPOTlight and RCTD pipelines. In

309   SPOTlight, a minimum cell-type proportion threshold is set to remove cell-types contributing

310   low amounts to pixels. To be consistent across methods, after deconvolution, cell-types in each

311   pixel whose proportions were less than the lowest ground truth pixel proportion for a cell-type

312   (2.5%) were removed, and the remaining cell-type proportions in a pixel were adjusted to sum to

313   1.

314   To compare the performance between STdeconvolve, SPOTlight, and RCTD, the root

315   mean squared error (RMSE) was computed for each pixel between the deconvolved and matched

316   ground truth cell-type proportions for each pixel in the ST dataset:

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317

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)^2}{n}}$$

318   where n is the number of cell-types, $\hat{y}_i$ is the predicted cell-type proportion for the cell-type i,

319   and y_i is the ground truth cell-type proportion for the cell-type i.

320

321   To compare the accuracy of each method deconvolving individual cell-types, the RMSE

322   between the predicted pixel proportions for a given cell-type and the matched ground truth cell-

323   type proportions across MERFISH ST dataset pixels was computed. Here, n is the number of

324   dataset pixels, $\hat{y}_i$ is the predicted cell-type proportion for the given cell-type in pixel i, and y_i is

325   the ground truth cell-type proportion for the given cell-type in pixel i. Because the RMSE scales

326   with abundance, the RMSE for each cell-type were divided by the standard deviation of the pixel

327   proportions for the corresponding ground truth-type to compare RMSEs across cell-types.

328

329   **Deconvolution of simulated MERFISH ST data using a reference with missing cell-types**

330   To simulate a single-cell reference with missing cell-types, cells annotated as "excitatory" and

331   "inhibitory" were removed from the previously constructed MERFISH single cell transcriptomic

332   profile reference and used to train SPOTlight and RCTD as described under `*Comparison to*

333   *SPOTlight and RCTD*`. The new trained models were then reapplied to the simulated ST

334   MERFISH dataset of 3072 pixels for deconvolution. After deconvolution, cell-types in each

335   pixel whose proportions were less than the lowest ground truth pixel proportion for a cell-type

336   (2.5%) were removed and the remaining cell-type proportions were adjusted to sum to 1, as

337   described previously. Pixel RMSEs were computed as described above based on the

338   deconvolved cell-type proportions and the ground truth dataset, which retained excitatory and

339   inhibitory neuronal cell-types.

340

341   **Deconvolution of ST data of the mouse olfactory bulb (MOB)**

342   Mouse olfactory bulb datasets were obtained from the original publication[9]. We focused on

343   MOB replicate #8, as the primary MOB ST dataset in this work. We first removed genes with

344   less than 100 reads detected across pixels and pixels with fewer than 100 total gene counts,

345   resulting in a cleaned dataset of 260 pixels and 7365 genes. Overdispersed genes were

346   determined were defined as genes with higher-than-expected observed expression variance

347   across the pixels[20]. Expression variance was modeled based on the expression magnitude using a

348   general additive model with a basis of 5. The p-value of a gene being overdispersed was

349   determined using the cumulative distribution function of the $\mathcal{X}^2$ distribution with degrees of

350   freedom equal to the number of pixels – 1. A gene was overdispersed if the multiple testing

351   adjusted p-value was < 0.05. For MOB replicate #8, we obtained 255 overdispersed genes. We

352   used STdeconvolve to fit LDA models with a range of integer Ks from 2 to 20 and chose the

353   model with $K=12$, which was within the range of K's that produced the lowest perplexity and the

354   number of "rare" cell-types with mean pixel proportion < 5% was 0 (Supplementary Figure

355   S6C). After deconvolution, cell-types in each pixel whose proportions were less than 5% were

356   removed and the remaining cell-type proportions in each pixel were adjusted to sum to 1.

357   Without a ground truth reference, this filtering threshold was based on the variable performance

358   of the different deconvolution methods to accurately deconvolve cell-types represented below

359   this pixel proportion (Supplementary Note 2).

360   For SPOTlight and RCTD, we used a previously generated scRNA-seq reference of the

361   MOB[14]. We retained only cells collected from untreated wildtype animals and the resulting

362   matrix encompassed 17709 cells representing 38 previously annotated cell-type clusters and raw

363   counts for 18560 genes. The trained models were then applied to deconvolve cell-types in the

364   cleaned MOB replicate #8 ST dataset of 260 pixels and 7365 genes. After deconvolution, cell-

365   types in each pixel whose proportions were less than 5% were removed and the remaining cell-

366   type proportions were adjusted to sum to 1.

367

368   **Deconvolution of ST data of the mouse olfactory bulb (MOB) using a reference with**

369   **missing cell-types**

370   To simulate a single-cell reference with missing cell-types, cells of the MOB scRNA-seq

371   reference that were part of "OEC" clusters 1-5 were removed. SPOTlight and RCTD were

372   trained using this new reference and the newly trained models were then reapplied to the cleaned

373   MOB replicate #8 ST dataset of 260 pixels and 7365 genes for deconvolution. After

374   deconvolution, cell-types in each pixel whose proportions were less than 5% were removed and

375   the remaining cell-type proportions were adjusted to sum to 1.

376

377   **Deconvolution of ST data of the mouse olfactory bulb (MOB) using cortex reference**

378   For the scRNA-seq reference of the mouse cortex, we used the scRNA-seq cortex dataset

379   provided by SPOTlight containing 1404 cells representing 23 transcriptionally distinct clusters

380   and 34617 genes. SPOTlight and RCTD were trained using this reference and the newly trained

381   models were then reapplied to the cleaned MOB replicate #8 ST dataset of 260 pixels and 7365

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382   genes for deconvolution. After deconvolution, cell-types in each pixel whose proportions were

383   less than 5% were removed and the remaining cell-type proportions were adjusted to sum to 1.

384

385   **Comparison between STdeconvolve, SPOTlight, and RCTD**

386   As neither RCTD nor SPOTlight returns transcriptional profiles, we compared methods by

387   evaluating the Pearson's correlation between the pixel proportions of each deconvolved cell-type

388   from any two methods. Cell-type clusters based on the MOB reference that were deconvolved by

389   RCTD or SPOTlight were matched to STdeconvolve cell-types that had the highest Pearson's

390   correlation.

391

392   **Deconvolution of ST data of breast cancer sections**

393   ST datasets of 4 breast cancer sections were obtained from the original publication[15]. Genes with

394   less than 10 reads across pixels or pixels with less than 10 total reads were removed from each

395   dataset and genes present in more than 95% of pixels for given dataset were removed.

396   Overdispersed genes were determined for each dataset as described in `*Deconvolution of ST data*

397   *of the mouse olfactory bulb (MOB)*` using the same parameters. After, we combined the 4 breast

398   cancer datasets into a single dataset of 1029 pixels with counts for 372 genes found to be

399   overdispersed in at least one dataset. We trained LDA models on this combined dataset with

400   STdeconvolve using a range of K from 2 to 20 and selected $K=15$, which was within the range of

401   K's that produced the lowest perplexity and the number of "rare" cell-types with mean pixel

402   proportion < 5% was 0 (Supplementary Figure S10C).

403

404   **Gene set enrichment analysis of deconvolved breast cancer cell-types**

405   To interpret the transcriptional profiles of the deconvolved cell-types in ST data of breast cancer

406   sections, we used gene set enrichment analysis as implemented in the `liger` R package[21]. We

407   filtered the list of 16771 Homo sapiens Gene Ontology gene set terms[23] to include those which

408   contained at least 1 gene present in the input ST dataset corpus used with STdeconvolve,

409   resulting in 4238 terms. We then performed iterative gene set enrichment analysis on the ranked

410   expression profile of the genes as previously described in `*Annotation and matching of*

411   *deconvolved and ground truth cell-types*`

412

Clustering analysis of ST pixels

414   For clustering analysis of the MOB, using the cleaned MOB replicate #8 ST dataset of 260 pixels

415   and 7365 genes, the raw counts were normalized to counts per million and adjusted to a $\log_{10}$

416   scale with pseudo count 1. Subsequently, dimensionality reduction using PCA was performed,

417   and pixels were visualized using 2-D embedding with t-SNE on the top 5 principal components

418   and perplexity = 30. Graph-based cluster detection using Louvain clustering[24] was performed

419   using the top 5 principal components with the maximum number of nearest neighbors equal to

420   30, resulting in the assignment of pixels to 5 clusters, which were manually annotated based on

421   the physical locations of the pixel clusters on the MOB tissue section[9].

422   For clustering analysis of the breast cancer sections, we took the combined dataset of

423   1029 pixels and 372 overdispersed genes, and $\log_{10}$ transformed with pseudo count of 1, and

424   dimensionality reduction using PCA was performed. In a manner similar to the original

425   publication, pixels were clustered into 3 groups using the "Ward.D" method and the Euclidean

426   distance calculated using the first 2 principle components This resulted in the assignment of

427   pixels to 3 clusters which corresponded to the annotations of the 3 histological sections

428   previously annotated by pathologists[15].

429

430   **Availability of Code**

431   STdeconvolve is available as an open-source R software package[25] with the source code

432   available in the Supplemental Material and on GitHub at https://github.com/JEFworks-

433   Lab/STdeconvolve. Additional documentation and tutorials are available at

434   https://jef.works/STdeconvolve/

435

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500

501

502   **Main Figures**

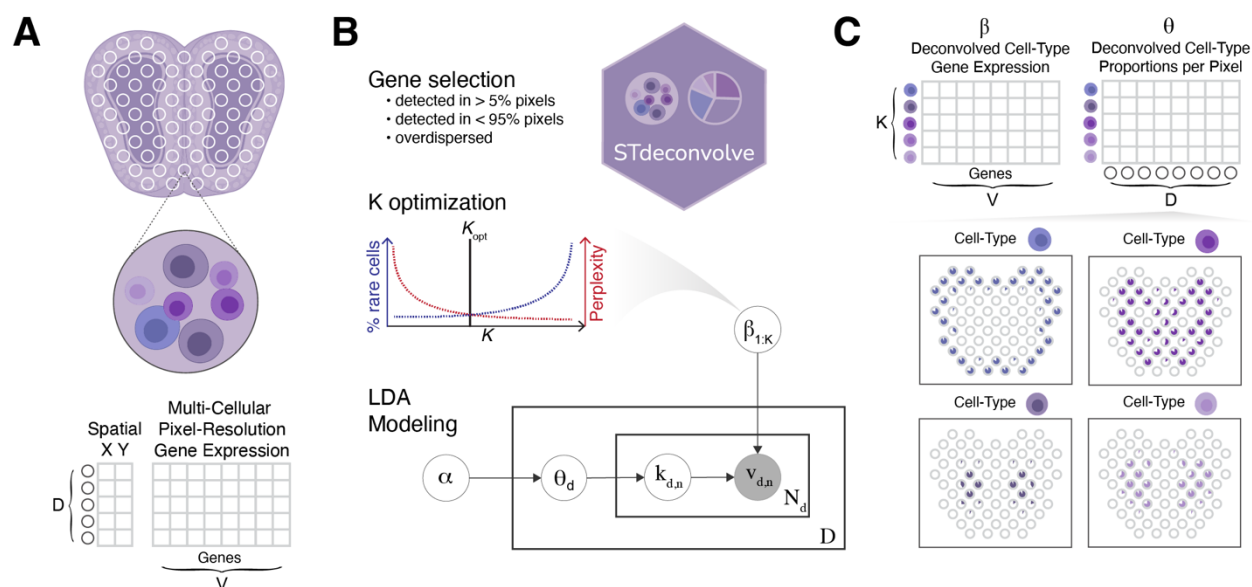504   Figure 1. Overview of STdeconvolve.

505   A. STdeconvolve takes as input a spatial transcriptomics (ST) dataset of D pixels (rows) and

506   counts of V genes (columns). A matrix of spatial coordinates for each of the D pixel can

507   also be used for visualization.

508   B. STdeconvolve first feature selects genes to retain in the input matrix for deconvolution,

509   such as genes with counts in more than 5% and less than 95% of the pixels, and

510   overdispersed across the pixels. To determine the optimal number of cell-types to be

511   deconvolved, K, STdeconvolve fits multiple LDA models to the input dataset each with a

512   different K. STdeconvolve computes the perplexity and number of rare deconvolved cell-

513   types to guide the selection of the model with the optimal K. A graph representation of

514   LDA modeling is shown, where $\beta_{1:K}$ is a $K \times V$ gene-probability matrix for each cell-

515   type k and each input matrix gene v; D is the number of pixels in the dataset; N_d is the

516   total gene counts in pixel d; α is the Dirichlet distribution scaling parameter; θ_d is the

517   multinomial distribution of cell-type proportions in pixel d drawn from $Dir(\alpha)$; $k_{d,n}$ is a

24

518   drawn cell-type from $mult(\theta_d)$ for the nth gene count in pixel d; $v_{d,n}$ is a count of gene v

519   drawn from gene-probability $mult(\beta_{k_{d,n}})$, given the cell-type $k_{d,n}$. (See Methods for

520   details). Shaded circle indicates observed variables and clear circles indicate latent

521   variables.

522 C. STdeconvolve outputs two matrices: (1) β, the deconvolved transcriptional profile matrix

523   of K cell-types over genes V, and (2) θ, the proportions of K cell-types across the D

524   pixels. The proportion of deconvolved cell-types can then be visualized across the pixels.
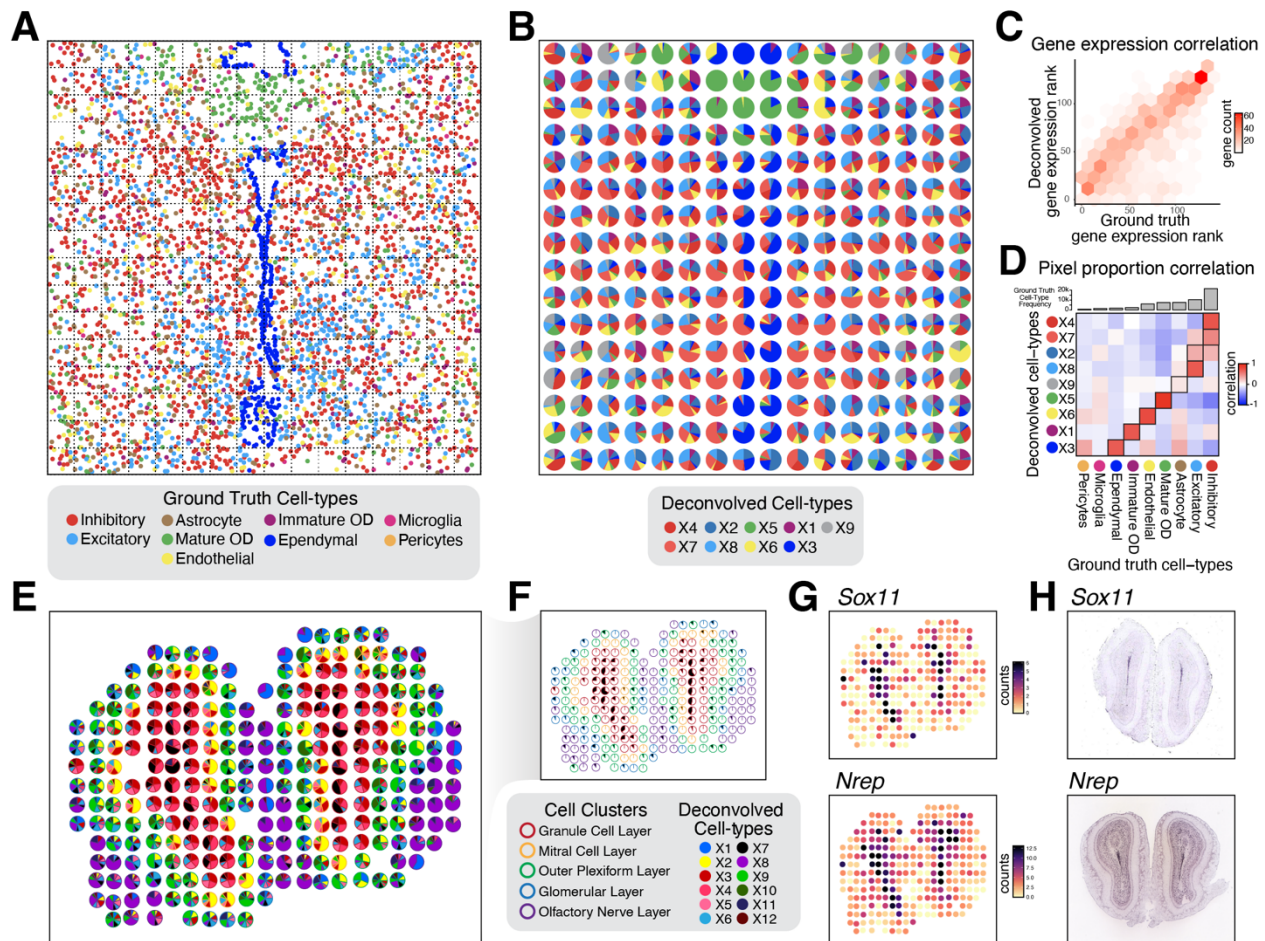
525

Figure 2. STdeconvolve characterizes the spatial organization of cell-types in simulated and real ST data.

A. Ground truth single-cell resolution MERFISH data of one section of the MPOA partitioned into 100 μm^2 pixels (black dashed squares). Each dot is a single cell colored by its ground truth cell-type label.

B. Proportions of deconvolved cell-types from STdeconvolve, represented as pie charts for each simulated pixel.

C. The ranking of each gene based on its expression level in the transcriptional profiles of the deconvolved cell-types, compared to its gene rank in the transcriptional profile of the matched ground truth cell-type.

537 D. Heatmap of Pearson's correlations between the proportions of the deconvolved cell-types

538   and ground truth cell-types across simulated pixels. Ground truth cell-types are ordered

539   by their frequencies in the ground truth dataset. Matched deconvolved and ground truth

540   cell-types are boxed.

541 E. Deconvolved cell-type proportions for ST data of the MOB from STdeconvolve,

542   represented as pie charts for each ST pixel. Pixels are outlined with colors based on the

543   pixel transcriptional cluster assignment corresponding to MOB coarse cell layers.

544 F. Highlight of deconvolved cell-type X7. Pixel proportion of deconvolved cell-type X7 are

545   indicated as black slices in pie charts. Pixels are outlined with colors as in E).

546 G. Gene counts in each pixel of the MOB ST dataset for deconvolved cell-type X7's select

547   top marker genes *Sox11* and *Nrep*.

548 H. Corresponding ISH images for deconvolved cell-type X7's top marker genes *Sox11* and
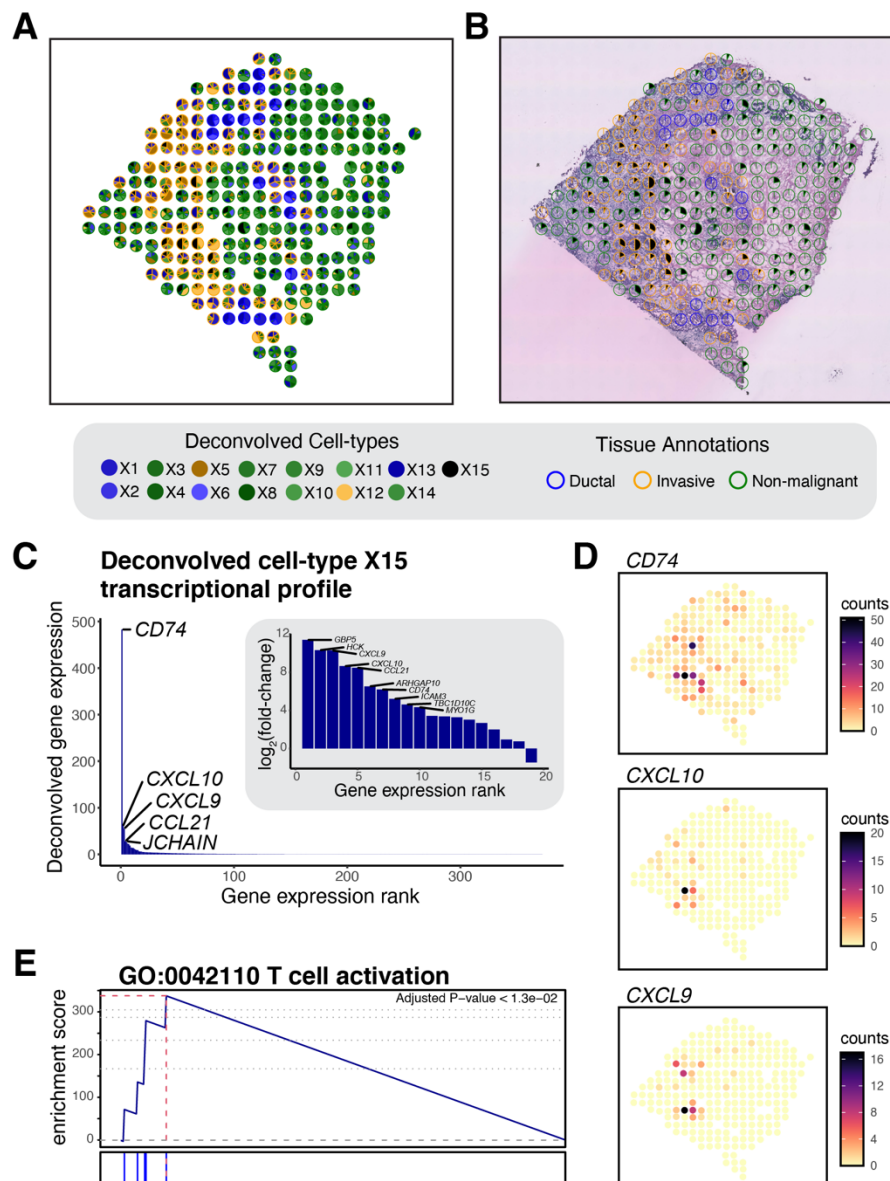
549   *Nrep* from the Allen Brain Atlas[13].

550

Figure 3. STdeconvolve characterizes the spatial organization of immune cells in breast cancer ST data.

A. Deconvolved cell-type pixel proportions for ST data of a breast cancer tissue section, represented as pie charts. Pixels are outlined with colors based on the pixel transcriptional cluster assignment corresponding to 3 pathological annotations.

557   B. Highlight of deconvolved cell-type X15. Pixel proportion of deconvolved cell-type X15

558   are indicated as black slices in pie charts. Pixels are outlined with colors as in A). An

559   H&E-stained image of the breast cancer tissue is shown in the background.

560   C. Barplot of the deconvolved transcriptional profile of deconvolved cell-type X15 ordered

561   by magnitude of deconvolved gene expression. Inset represents the $\log_2$ fold-change of

562   the deconvolved transcriptional profile genes with respect to the mean expression of the

563   other 14 deconvolved cell-type transcriptional profiles. Select highly expressed and high

564   fold-change genes are labeled.

565   D. Gene counts in each pixel of the breast cancer ST dataset for deconvolved cell-type

566   X15's select top marker genes.

567   E. Gene set enrichment plot for significantly enriched GO term "T cell activation" for

568   deconvolved cell-type X15's transcriptional profile.

569