1 **eSPRESSO: a spatial self-organizing-map clustering method for** 2 **single-cell transcriptomes of various tissue structures using** 3 **graph-based networks**

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30

31 **ABSTRACT**

32 Animal cells are spatially organized as tissues and cellular gene expression data contain such
33 information that governs body structure and morphogenesis during developmental process. Although
34 several computational tissue reconstruction methods using transcriptomic data have been proposed,
35 those methods are insufficient with regard to arranging cells in their correct positions in tissues or
36 organs unless spatial information is explicitly provided. Here, we propose eSPRESSO, a powerful *in*
37 *silico* three-dimensional (3D) tissue reconstruction method using stochastic self-organizing map
38 (stochastic-SOM) clustering, together with optimization of gene set by Markov chain Monte Carlo
39 (MCMC) framework, to estimate the spatial domain structure of cells in any topology of tissues or
40 organs from only their transcriptome profiles. We confirmed the performance of eSPRESSO by
41 mouse embryo, embryonic heart, adult cortical layers, and human pancreas organoid with high
42 reproducibility (success rate = 72.5–100%), while discovering morphologically important spatial
43 discriminator genes (SDGs). Furthermore, we applied eSPRESSO to analysis of human adult heart
44 diseases by virtual gene knockouts, and revealed candidate mechanisms of deformation of heart
45 structure. The eSPRESSO may provide novel methods to analyze the mechanisms of 3D structure
46 formation and morphology-based disease mechanisms.

47 INTRODUCTION

48 Analysis of disease mechanisms based on high-throughput single-cell RNA-sequencing is becoming
 49 prominent and fundamental technique in cell biology¹⁻³. Particularly, methods for high-throughput,
 50 spatially resolved single-cell RNA-seq have been developed and attracting attentions as novel
 51 analytics for disease mechanisms. Single-molecule fluorescence *in-situ* hybridization (FISH)⁴ has
 52 been widely used to quantitate transcript numbers at single-cell resolution, often within the context
 53 of a diseased tissues of interest. More recently, highly-multiplexed methods such as seqFISH⁵ or
 54 merFISH⁶ are reported to detect transcript dynamics of target cells in 3D location. More directly,
 55 high-resolved, 2D-mapped primer-based RNA-sequencing of tissues fixed on a glass plate has been
 56 also developed for mapping of transcript abundance, utilized to draw spatial cellular location map
 57 after reconstructing 3D image by multiple 2D-maps⁷. However, these methods are still in the early
 58 stage and requires further research and development in terms of practical cost and conveniences.

59 Alternatively, several computational methods to reconstruct 3D tissues by estimating the
 60 spatial positions of individual cells in tissues with gene expression data obtained by single-cell
 61 RNA-seq have been reported.⁸⁻¹⁴ These methods may be roughly divided into two types: the
 62 landmark approach and the *ab initio* approach. The landmark approach estimates the 3D position of
 63 each cell based on gene expression profiles while using the spatial information of marker genes
 64 obtained by *in situ* hybridization.⁸⁻¹⁰ Conversely, the *ab initio* approach assigns each cell to 3D space

65 according to the principal component score calculated from gene expression profiles without using
66 such spatial reference data.^{11–14} Although current principal component analysis (PCA)-based
67 methods are simply used for 3D visualization, an *ab initio* approach that does not depend on the
68 spatial information of marker genes obtained by *in situ* hybridization is promising for 3D
69 reconstruction.

70 Previously, we reported a novel 3D reconstruction method using stochastic-SOM
71 clustering, or SPRESSO (SPatial REconstruction by Stochastic-SOM)¹⁵, which features gene
72 selections based on Gene Ontology (GO).^{16,17} The method yielded high success rates of 3D
73 reconstructions of mouse gastrula embryo and demonstrated a remarkable ability to find SDGs that
74 contribute to differentiation and tissue morphogenesis. This method, however, was preliminary and
75 simply projected four domains of mouse gastrula to a cubic structure and thus unapplicable to more
76 complicated structures of organs such as heart or pancreas.

77 In this paper, we introduced graph-based SOM clustering to reconstruct any topology of
78 domains or tissues as long as they can be drawn as some kind of connection graphs or network
79 diagrams. The concept of graph-based SOM was already reported in 1989 by T. Kohonen and
80 colleagues¹⁸ who introduced a Kohonen map or network in 1982¹⁹, which is one of the artificial
81 neural networks and computationally convenient abstraction building on biological models of
82 neuronal systems²⁰ and morphogenesis models by Alan Turing²¹. So far, many useful topology

83 structures other than square grids such as toroidal grids that have opposite edges are connected²²
84 have been introduced. However, graph-network representation is the most simple but comprehensive
85 for describing relationships tissue domains. Thus, we applied graph-SOM to various types of mouse
86 and human organs that show specific topologies of tissue domain relationships. We also successfully
87 visualized the reconstructed relationships by uniform manifold approximation and projection
88 (UMAP)²³ to confirm the original structure.

89

90 **RESULTS**

91 All the dataset used in this paper is summarized in Table 1 and the detailed results are shown in
92 Supplementary Figures and Tables.

93 **Spatial clustering of gene expression data by graph-based SOM with gene set optimization**

94 Inspired by Kohonen’s self-organizing-map learning theory¹⁹, here we extended our previously
95 developed method, SPRESSO¹⁵, to graph-based networks, which is theoretically applicable to
96 cell-to-cell relationships of any types of topological structures of tissues or organs. The basic
97 algorithm of spatial clustering of cells is a combinatorial optimization to find best gene sets to
98 reproduce known topological network structures of learning objects, or gene vectors of cells. Since
99 each node on the topological network may contain multiple such gene vector objects, given an
100 original topological network, we can calculate the reproducibility of the structure by counting
101 correct and incorrect network edges, either connected or unconnected, after learning process. We
102 also used the Markov-Chain-Monte-Carlo (MCMC) to optimize the best gene sets to give a
103 maximum reproducibility of a topology of structure. We have also tuned the SOM learning process
104 by introducing stochastically learning (i.e., stochastic-SOM¹⁵) that allows learning efficiency in later
105 phase where the extent of learning ability usually decreases monotonously. We refer to this method
106 as eSPRESSO (enhanced-SPRESSO) in this paper.

107 Here we first tested the ability of eSPRESSO with mouse E5.5-7.5 gastrula embryo, whose

108 topological structures are too complicated to be handled and reproduced by our previous SPRESSO,
 109 by generating graph structure of 7 domains (Ect1, Ect2, Ect3, PS, MA, MP, E1-E2-E3) with
 110 domain-to-domain contact relationships (Fig.1a). We optimized the gene set to maximize the
 111 reproducibility of the original graph structure by 1,000 steps of MCMC calculations. To enhance the
 112 efficient optimization and stable reproducibility, we performed parallel tempering²⁴ based on 8
 113 parallel MCMC processes. The resulted clustering is evaluated with the original structure by simply
 114 counting the existing edges between two gene vector objects, or cells. We used gene expression data
 115 from 213 sections and the reproduced accuracy was 86.3% after the MCMC optimization and the
 116 domain contact map (Fig.1b) of samples for original (lower triangle) and reconstructed (upper
 117 triangle) structures (Fig.1c) are shown. A heatmap of gene expression data for optimized SDGs
 118 (Fig.1d) is also shown. The final clustering using SDGs is drawn by UMAP (Fig.1e).

119

120 **Circular and hierarchical structure reconstruction by eSPRESSO**

121 To evaluate the performance of eSPRESSO, we applied the method to circular and hierarchical
 122 structures of tissues. The development of mouse heart initiates with an expansion of a blood vessel,
 123 forming complex structure consisting of arteries and veins in later phase. We used scRNA-seq data
 124 from mouse embryonic heart structure (E9.25) which represents pseudo-circular structure of seven
 125 domains, of which five are projecting from the anterior and posterior second heart fields (AHF and

126 pSHF) (Fig.2a). When we down-sampled 10 cells for each domain from the initial 3911 cells and
 127 optimized the gene set by stochastic-SOM with MCMC to reproduce the original structure, we
 128 successfully reconstructed the topology of cellular locations with extremely high accuracy of 96.8%
 129 (Fig.2b). The number of final SDGs is 18 which contains many known important genes such as
 130 *Hand2*, *Gata4*, etc.²⁵, in mouse heart development. The visual projection by UMAP clustering of all
 131 3331 cells using the SDGs show a circular representation of tissue domains which is quite consistent
 132 of original structure (Fig.2a).

133 Then we further tried to reconstruct mouse liver structure where a nine layers of tissues
 134 from a concentric circle are observed¹⁰ (Fig.2c). The eSPRESSO correctly reconstructed the
 135 relationships of 90 cells, which are randomly selected by down-sampling 10 cells from each of 9
 136 domains, with 84.6% accuracy. The resulted 18 SDGs represent many known liver-specific genes
 137 such as cytochrome P450 (*Cyp1a2* and *Cyp2f2*) and hepatic nuclear factor 4 alpha (*Hnf4a*)²⁷ (Fig.2d).
 138 The visual projection by UMAP clustering of all 1415 cells using the 18 SDGs indicate that there are
 139 clear layer structure observed although it does not reproduce the circular structure due to the
 140 limitation of UMAP clustering that are difficult to generate circular layer topology (Fig.2c).

141

142 **Developmental analysis of human pancreas by eSPRESSO**

143 One of the powerful characteristics of eSPRESSO may be demonstrated by analysis of organs during

developmental process. We used scRNA-seq data from human developmental pancreas from stage 4, 5 and 6 data²⁸, and reconstructed the structure. The accuracies are high for all the stages such as 96-100%. The numbers of resulted SDGs are 9, 22, and 18, containing pancreas-specific genes such as insulin (*INS*) and insulin gene enhancer protein ISL-1 (*ISL1*)²⁹ (Supplementary Table1). Interestingly, although we did not input information of endocrine and non-endocrine separation, the UMAP plots of all the thousands of cells using the SDGs indicate that developmental events such as alpha-cell differentiation in Langerhans islets and exocrine cell detachment from endocrine cells are observed (Fig.3a-c).

In the stage 4 to 6 reconstructions, the original scRNA-seq data is focusing on endocrine cells; therefore, we obtained endocrine-related genes during stages. GSEA analysis also represent that the developmental changes of SDGs, indicating that more polarization between endo- and exocrine cells seemed to proceed.

Application to virtual human gene knockout experiment

One of the most useful usages of eSPRESSO may be to perform virtual *in-silico* human gene knockout experiments. As an example of organs, we used scRNA-seq data from human developmental heart³⁰. We first successfully reconstructed the 8 domain structure with accuracy of 76.2% for postconceptional weeks (PCWs) 4.5-5 human embryonic heart (Fig.4a). We obtained ##

162 SDGs as the optimized result (Supplementary Table1). Then we performed single gene deletions
 163 among the SDGs to see if any structural changes are observed (Fig.4b). Among the 37 SDGs, most
 164 effective genes that dramatically decreases accuracies of reconstructions are found (Supplementary
 165 Table2). The structural change for gene X deletion is shown in Fig.4c. The gene X is the orthologous
 166 gene in mouse that is homozygous lethal. The decreases of accuracies are also shown
 167 (Supplementary Table2). All other structural changes are shown in Supplementary Figure 1. We also
 168 performed two gene deletions (Supplementary Table3).

169 We also performed similar analyses for scRNA-seq data from PCW 6.5 and 9 human
 170 hearts. The most effective genes that decreases accuracies of reconstructions are shown in
 171 Supplementary Table 4.

172

173 **Discussion**

174 In this paper, we developed a computational approach that combines the SOM
175 clustering under the topological constraints and gene set optimization by MCMC. The
176 reproducibility of tissue topology is sufficiently high and this method may innovatively
177 add more information on the existing clustering method such as helping to find spatially
178 distributed discriminator genes (SDGs) and inference of developmental architecture of
179 tissue domains. It is also useful to investigate the collapses of spatial clustering by
180 *in-silico* virtual gene knockouts although it is not a real knock-out experiments.

181 Recently, alternative direct experimental approaches such as highly-multiplexed
182 FISH or direct spatial transcriptome have been proposed. However, the primal purpose
183 of these methods is to reconstruct cellular locations with gene expression data. Although
184 they can contribute to detect differentially expressed genes among distinct tissue
185 domains, it does not explicitly consider the tissue topologies and thus, it may lack to
186 detect spatially contributing genes in terms of neighboring tissue networks. Our method
187 may be able to complement such a neighboring tissue information and it is also
188 applicable to the results of such data to detect globally expressed genes that contributes
189 to domain networks in spatial distributing manner

190 Our methods may have benefits in analysis of organ development. Many genes

191 show homozygous lethality in development. Although many evidences based on animal
192 studies indicate that there are candidates of those lethal genes in human, it is not
193 possible to reproduce lethality in human system due to ethical reasons. The human
194 organoids derived from iPS or ES cells may provide an alternative approach to
195 reproduce gene knockout organs. However, abnormalities are often stochastic and
196 affected by other internal or external factors and it sometimes requires a large number of
197 repeat experiments to reproduce the phenomena³¹. Contrastingly, our *in-silico* virtual
198 knockout method does not require any cumbersome protocols and may be able to
199 reproduce the same results in many times.

200 The SOM learning is a renowned method in the field of clustering, first
201 proposed by Kohonen and colleagues¹⁹. They have already introduced graph-network
202 topology for SOM clustering but there existed not many examples to solve in their
203 days¹⁸. The original SOM learning decreases learning regions and efficiency
204 monotonously, which causes clustering problems in the later phase and we found that
205 efficient scheduling for learning was necessary for practical application to the current
206 single-cell transcriptome datasets. Thus, we introduced the stochastic-SOM learning
207 which has a characteristic similar to that of Gibbs-sampling although the sampling
208 probability is totally equal. This improvement dramatically enhanced the clustering

209 ability and thus enabled to implement this study.

210 Finally, we state that our current algorithms and analysis pipeline describe here
 211 is still primitive and many modifications are necessary. The extraction of initial gene
 212 candidates are solely dependent on Boruta package that detects feature genes by random
 213 forest generation and may miss important genes that overlaps categories in the distant
 214 branches or leaves. The parallel tempering MCMC is also a suboptimal approach,
 215 although it enhances the combinatorial optimization to allow quick convergence to the
 216 global optimum, which sometimes fail to find the best gene set to reproduce the
 217 topology by SOM clustering. We expect that more efficient and fine-grained algorithms
 218 to find global optimum in decent time may replace modules in our pipeline in future.

219

220 **Methods**

221 **Methods and any associated references are available in the online version of the paper.**

222 **Note: Any Supplementary Information and Source Data files are available in the online**
 223 **version of the paper.**

224

225 **Code and data availability**

226 The proposed methods including the feature gene selection, the 3D reconstruction using
 227 stochastic-SOM clustering, and visualization, are implemented in R and Python and are available at
 228 <https://github.com/tmorikuicr/espresso>. Also all the gene expression data used in this study is
 229 included together.

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304

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310

311 **Author contributions**

312 W.F. conceptualized and designed the study. T.M. implemented the software. T.M., K.L. and T.T.
313 performed the computational experiments. J.Yamane., C.A., K.O., and J.Yamashita. performed the
314 data interpretation from a biological point of view. T.M. and W.F. wrote the manuscript. All authors
315 have read and approved the final manuscript.

316

317 **Competing Interests**

318 The authors declare no competing interests.

319 **Figure Legends**

320 **Figure 1. Overview of the 3D reconstruction method of mouse embryo using stochastic-SOM**
 321 **clustering with graph-network topology.**

322 **Figure 2. Examples of 3D reconstructions: a,b) mouse developmental heart and c,d) mouse**
 323 **liver.**

324 **Figure 3. Example of 3D reconstructions during pancreas development.**

325 **Figure 4. Example of virtual knockout analysis by eSPRESSO in human developmental heart.**

Data Name	Reference	#Domain	#Cells	#Initial genes	#SDGs	Max Accuracy	ARI
Mouse embryo (E7.0)	Peng, Guangdun, et al. "Spatial transcriptome for the molecular annotation of lineage fates and cell identity in mid-gastrula mouse embryo." <i>Developmental cell</i> 36.6 (2016): 681-697.	4	41(sections)	185	18	100.0%	1
Mouse embryo (E5.5-7.5)	Peng, Guangdun, et al. "Molecular architecture of lineage allocation and tissue organization in early mouse embryo." <i>Nature</i> 572.7770 (2019): 528-532.	7	213 (sections)	87	17	86.3%	0.79731
Mouse brain (ALM)	Tasic, Bosiljka, et al. "Shared and distinct transcriptomic cell types across neocortical areas." <i>Nature</i> 563.7729 (2018): 72-78.	3	3809	106	7	100.0%	1
Mouse brain (VISp)		4	7049	122	19	100.0%	1
Mouse heart (E7.75)	de Soysa, T. Yvanka, et al. "Single-cell analysis of cardiogenesis reveals basis for organ-level developmental defects." <i>Nature</i> 572.7767 (2019): 120-124.	4	1259	51	9	100.0%	1
Mouse heart (E8.25)		7	3331	132	22	97.5%	0.93082
Mouse heart (E9.25)		7	3911	93	18	96.8%	0.92909
Mouse liver	Halpern, Keren Bahar, et al. "Single-cell spatial reconstruction reveals global division of labour in the mammalian liver." <i>Nature</i> 542.7641 (2017): 352-356.	9	1415	97	18	84.6%	0.48085
Human pancreas (S4)	Veres, Adrian, et al. "Charting cellular identity during human in vitro β -cell differentiation." <i>Nature</i> 569.7756 (2019): 368-373.	5	5273	107	21	95.9%	0.86908
Human pancreas (S5)		5	3925	51	21	97.6%	0.94836
Human pancreas (S6)		5	5051	56	18	95.5%	0.853
Human heart (4.5-5PCW)	Asp, Michaela, et al. "A spatiotemporal organ-wide gene expression and cell atlas of the developing human heart." <i>Cell</i> 179.7 (2019): 1647-1660.	8	238 (spots)	57	37	76.2%	0.5314551
Human heart (6.5PCW)		8	1515 (spots)	108	15	70.6%	0.3526241
Human heart (9PCW)		8	1358 (spots)	101	24	72.5%	0.3513529

Table 1. Datasets and performances of 3D structure reconstructions (score = accuracy + ARI)

Figure 1

(a)

Graph-SOM

Feature genes

Combinatorial Optimization of Genes
(Markov Chain Monte Carlo)

(b)

(d)

(c)

(e)

Figure 2

(a)

Mouse developmental heart

(b)

(c)

Mouse liver

(d)

Figure 3

Human developmental pancreas (stage 4 to 6)

(a)

(b)

(c)

istance of sample cells in original (lower) and after (upper) GraphSOM

istance of sample cells in original (lower) and after (upper) GraphSOM

istance of sample cells in original (lower) and after (upper) GraphSOM

Figure 4

Human developmental heart

(a)

(b)

VKO analysis

(c)

Before -> After