

1 VIA: Generalized and scalable trajectory inference in single-cell omics data

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7 Abstract

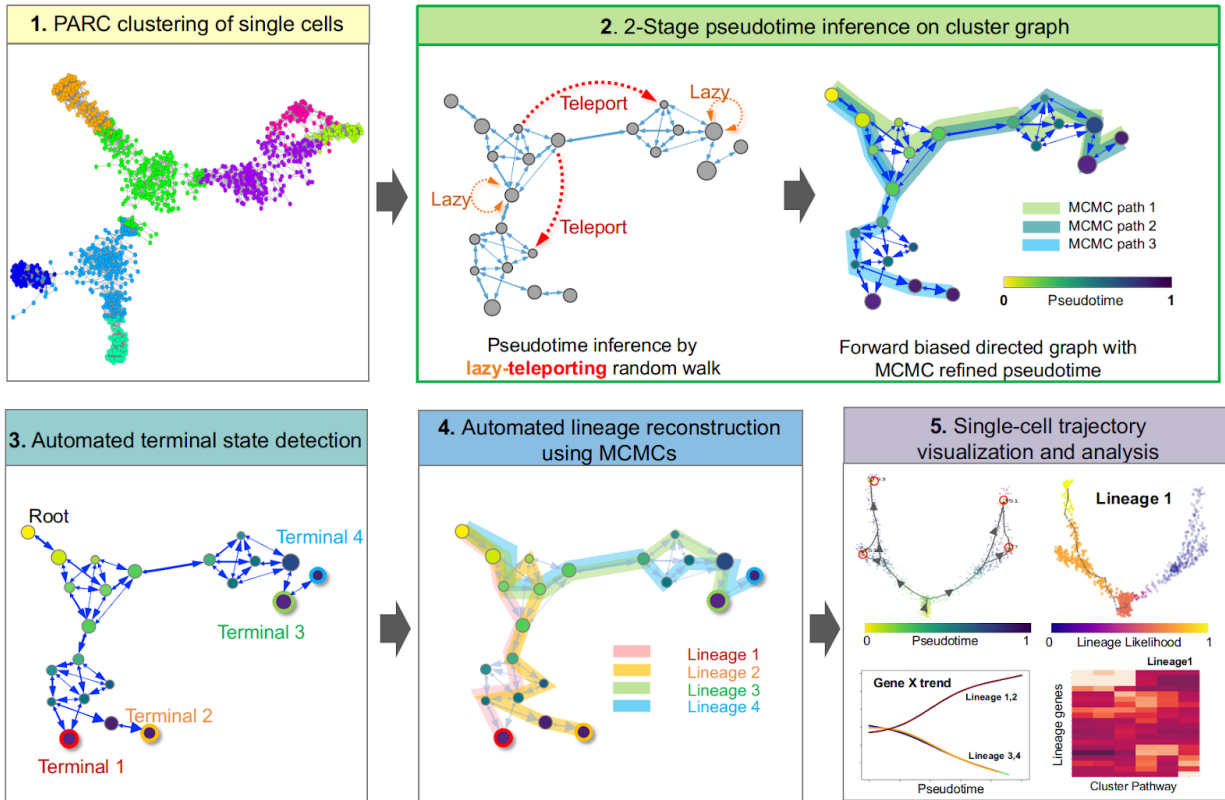
8 Inferring cellular trajectories using a variety of omic data is a critical task in single-cell data science.
9 However, prediction and thus biologically meaningful discovery of cell fates are challenged by the sheer
10 size of single-cell data, diverse omic data types, and their complex data topologies. We present VIA, a
11 scalable trajectory inference algorithm that uses lazy-teleporting random walks to accurately reconstruct
12 complex cellular trajectories beyond tree-like pathways (e.g. cyclic or disconnected structures), and to
13 discover less populous lineages or those otherwise obscured in other methods. VIA outperforms existing
14 algorithms in recapitulating cell fates/lineages, and also mitigates loss of global connectivity information
15 in large datasets beyond a million cells. Furthermore, VIA demonstrates versatility by distilling cellular
16 trajectories in single-cell transcriptomic, epigenomic, proteomic and morphological data – showing new
17 promise in scalable, multifaceted single-cell analysis to explore novel biological processes.

18 Introduction

19 Single-cell omics data captures snapshots of cells that catalog cell types and molecular states with high
20 precision. These high-content single-cell readouts can be harnessed to model evolving cellular
21 heterogeneity and track dynamical changes of cell fates in tissue, tumour, and cell population. However,
22 current computational methods face four critical challenges. First, it remains difficult to accurately
23 reconstruct high-resolution cell trajectories and detect cell fates embedded within them. Even the few
24 algorithms which automate cell fate detection (e.g., SlingShot¹ and Palantir²) exhibit low sensitivity and
25 are highly susceptible to changes in input parameters. Second, current trajectory inference (TI) methods
26 predominantly work well on tree-like trajectories (e.g. Slingshot, Monocle²³), but lack the generalisability
27 to infer disconnected, cyclic or hybrid topologies without imposing restrictions on transitions and
28 causality⁴. Third, the growing scale of single-cell data, notably cell atlases of whole organisms^{6,7},
29 embryos^{8,9} and human organs¹⁰, exceeds the existing TI capacity, not just in runtime and memory, but in
30 preserving global connectivity, which is often lost after extensive dimension reduction or subsampling.
31 Fourth, fueling the advance in single-cell technologies is the ongoing pursuit to understand cellular
32 heterogeneity from a broader perspective beyond transcriptomics. However, the applicability of TI to a
33 broader spectrum of single-cell data has yet to be fully exploited.

34 To overcome these recurring challenges, we present VIA, a graph-based TI algorithm that uses a new
35 strategy to compute pseudotime, and reconstruct cell lineages based on lazy-teleporting random walks
36 integrated with Markov chain Monte Carlo (MCMC) refinement. VIA relaxes common constraints on
37 traversing the graph by allowing cyclic and temporally reversed movements, and thus robustly detects cell
38 fates involving complex transitions that are otherwise obscured in other methods. VIA outperforms
39 popular TI algorithms in terms of capturing cellular trajectories not limited to multi-furcations and trees,
40 but also disconnected and cyclic topologies (**Supplementary Fig. S1**). Compared to existing TI methods,

41 VIA is highly scalable with respect to number of cells (10^2 to $>10^6$ cells) and features, without requiring
 42 extensive dimensionality reduction or subsampling which compromise global information. We
 43 demonstrate VIA's accuracy, scalability, topological-generalizability and multi-omic versatility across
 44 multiple modalities by investigating 10 simulated and experimental datasets (**Supplementary Table S1**),
 45 ranging from single-cell RNA-sequencing (scRNA-seq), single-cell sequencing assay for
 46 transposase-accessible chromatin (scATAC-seq), multi-omics integration, to mass and imaging cytometry.



47 **Figure 1. General workflow of VIA algorithm.** **Step 1:** Single-cell level graph is clustered such that each node
 48 represents a cluster of single cells (computed by our clustering algorithm PARC¹¹). The resulting cluster graph forms
 49 the basis for subsequent random walks. **Step 2:** 2-stage pseudotime computation: (i) The pseudotime (relative to a
 50 user defined start cell) is first computed by the expected hitting time for a lazy-teleporting random walk along an
 51 undirected graph. At each step, the walk (with small probability) can remain (orange arrows) or teleport (red arrows)
 52 to any other state. (ii) Edges are then forward biased based on the expected hitting time (See forward biased edges
 53 illustrated as the imbalance of double-arrowhead size). The pseudotime is further refined on the directed graph by
 54 running Markov chain Monte Carlo (MCMC) simulations (See 3 highlighted paths starting at root). **Step 3:** Consensus
 55 vote on terminal states based on vertex connectivity properties of the directed graph. **Step 4:** lineage likelihoods
 56 computed as the visitation frequency under lazy-teleporting MCMC simulations. **Step 5:** visualization that combines
 57 network topology and single-cell level pseudotime/lineage probability properties onto an embedding using GAMs, as
 58 well as unsupervised downstream analysis (e.g. gene expression trend along pseudotime for each lineage).

59 Results

60 Algorithm

61 VIA first represents the single-cell data as a cluster graph (i.e. each node is a cluster of single cells),
62 computed by our recently developed data-driven community-detection algorithm, PARC, which allows
63 scalable clustering whilst preserving global properties of the topology needed for accurate TI¹¹ (**Step 1 in**
64 **Fig. 1**). The cell fates and their lineage pathways are then computed by a two-stage probabilistic method,
65 which is the key algorithmic contribution of this work (**Step 2 in Fig. 1**, see **Methods**). In the first stage,
66 VIA models the cellular process as a modified random walk that allows degrees of *laziness* (remaining at
67 a node/state) and *teleportation* (jumping to any other node/state) with pre-defined probabilities. The
68 pseudotime, and thus the graph directionality, can be computed based on the theoretical hitting times of
69 nodes (See the theory and derivation in **Methods and Supplementary Note 2**). The lazy-teleporting
70 behavior prevents the expected hitting time from converging to a local distribution in the graph as
71 otherwise occurs in regular random walks, especially when the sample size grows¹². More specifically, the
72 laziness and teleportation factors regulate the weights given to each eigenvector-value pair in the expected
73 hitting time formulation such that the stationary distribution (given by the local-node degree-properties in
74 regular walks) does not overwhelm the global information provided by other ‘eigen-pairs’. Moreover, the
75 computation does not require subsetting the first k eigenvectors (bypassing the need for the user to select
76 a suitable threshold or subset of eigenvectors) since the dimensionality is not on the order of number of
77 cells, but equal to the number of clusters. Hence all eigenvalue-eigenvector pairs can be incorporated
78 without causing a bottleneck in runtime. Consequently in VIA, the modified walk on a cluster-graph not
79 only enables scalable pseudotime computation for large datasets in terms of runtime, but also preserves
80 information about the global neighborhood relationships within the graph. In the second stage of Step 2,
81 VIA infers the directionality of the graph by biasing the edge-weights with the initial pseudotime
82 computations, and refines the pseudotime through MCMC simulations. Next (**Step 3 in Fig. 1**), the
83 MCMC-refined graph-edges of the lazy-teleporting random walk enable accurate predictions of terminal
84 cell fates through a consensus vote of various vertex connectivity properties derived from the directed
85 graph. The cell fate predictions obtained using this approach are more robust to changes in input data and
86 parameters compared to other TI methods (**Supplementary Fig. S1 and Fig. S16**). Trajectories towards
87 identified terminal states are resolved using lazy-teleporting MCMC simulations (**Step 4 in Fig. 1**). The
88 probabilistic approach and relaxation of edge constraints allowed by VIA in computing differentiation
89 pathways and pseudotime enables greater sensitivity to cell fates and complex trajectories, and makes
90 allowances for asynchrony in differentiation processes by avoiding prematurely imposing constraints on
91 node-to-node mobility. Other methods resort to constraints such as reducing the graph to a tree, imposing
92 unidirectionality by thresholding edges based on pseudotime directionality, removing outgoing edges
93 from terminal states^{13,2} and computing shortest paths for pseudotime^{2,1}. VIA’s probabilistic approach to
94 graph-traversal allows it to infer cell fates when the underlying data spans combinations of multifurcating
95 trees and cyclic/disconnected topologies - topologies and hence lineages often obscured in existing TI
96 methods (**Supplementary Fig. S1**). Together, these four steps facilitate holistic topological visualization
97 of TI on the single-cell level (e.g. using UMAP or PHATE embeddings^{14,15}) and other data-driven
98 downstream analyses such as recovering gene expression trends (**Methods**). (**Step 5 in Fig. 1**).

99 VIA accurately infers trajectories in diverse scRNA-seq data

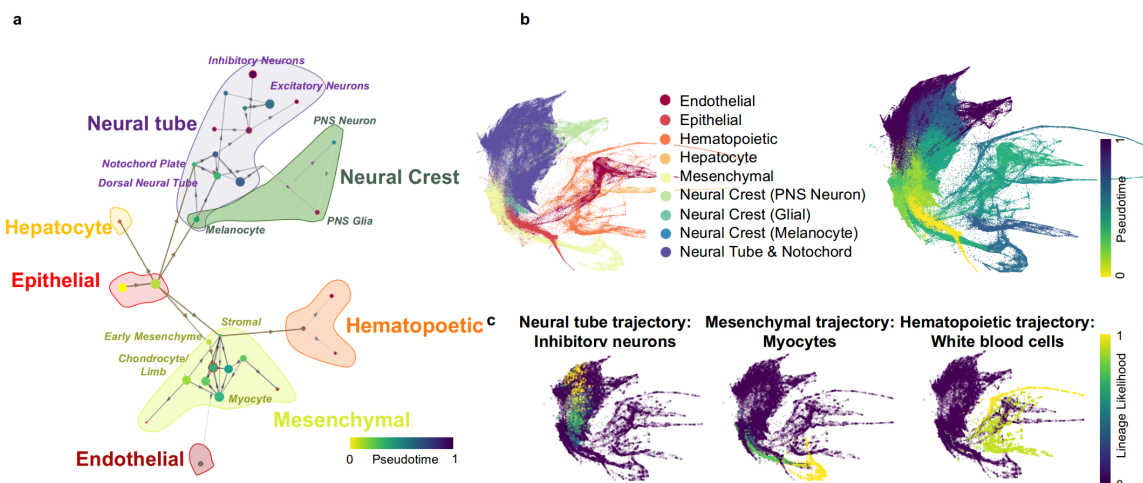
100 VIA recapitulates differentiation topologies and identifies elusive cell fates across a wide range of
101 transcriptomic data. We first showcase the ability of VIA to explore large single-cell transcriptomic
102 datasets by employing the 1.3-million-cell mouse organogenesis cell atlas (MOCA)⁸. While this dataset is
103 inaccessible to most TI methods from a runtime and memory perspective, VIA can efficiently resolve the
104 underlying developmental heterogeneity, including 9 major trajectories (**Fig. 2a,b**) with a runtime of ~40
105 min, compared to the next fastest method which has a runtime of at least 4 hours² (**Supplementary Table**
106 **S3**). VIA preserves wider neighborhood information and reveals a globally connected topology of MOCA
107 which is otherwise lost in the previous method. Broadly speaking, the overall cluster graph of VIA
108 consists of three main branches that concur with the known developmental process at early
109 organogenesis.¹⁶ (**Fig. 2a**). It starts from the root stem which has a high concentration of E9.5 early
110 epithelial cells made of multiple sub-trajectories (e.g. epidermis, nose and foregut/hindgut epithelial cells
111 derived from the ectoderm and endoderm). The stem is connected to two distinct lineages: 1)
112 mesenchymal cells originated from the mesoderm which arises from interactions between the ectoderm
113 and endoderm¹⁷ and 2) neural tube/crest cells derived from neurulation when the ectoderm folds inwards¹.
114 The sparsity of early cells (only ~8% are E9.5) and the absence of earlier ancestral cells make it
115 particularly challenging to capture the simultaneous development of trajectories. However, the overall
116 pseudotime structure presented by VIA is reasonable. For instance, at the junction of the
117 epithelial-mesenchymal branch, we find early mesenchymal cells from E9.5-E10.5. Cells from later
118 mesenchymal developmental stages (e.g. myocytes from E12.5- E13.5) reside at the leaves of branches.
119 Similarly, at the junction of epithelial-neural tube, we find dorsal tube neural cells and notochord plate
120 cells which are predominantly from E9.5-E10.5 and more developed neural cells at the tips (e.g.
121 excitatory and inhibitory neurons from E12.5-E13.5). VIA also places the other dispersed groups of
122 trajectories (e.g. endothelial, hematopoietic) in biologically relevant neighborhoods (**Supplementary**
123 **Notes 3, Supplementary Fig. S11**). While VIA's connected topology offers a coarse-grained holistic
124 view, it does not compromise the ability to delineate individual lineage pathways (consistent with those
125 found by Cao et al.,⁸) as shown in **Fig. 2c and Supplementary Fig. S11**. TI using VIA uniquely
126 preserves both the global and local structures of the data and is thus particularly favorable for biological
127 exploration involving large datasets, especially for comparative studies involving cell atlases¹⁹. Whilst
128 manifold-learning methods are often used to extensively reduce dimensionality to mitigate the
129 computational burden of large single-cell datasets, they tend to incur loss of global information and be
130 sensitive to input parameters. VIA is sufficiently scalable to bypass such a step, and therefore retains a
131 higher degree of neighborhood information when mapping large datasets. This is in contrast to
132 Monocle3's⁸ UMAP-reduced inputs that reveal different disconnected super-groups and fluctuating
133 connectivity depending on input parameters (see **Supplementary Fig. S12-15** for the biologically
134 consistent structures proposed by VIA across a range of parameters compared to the contradicting cell
135 super groups and connectivity suggested by a UMAP based TI interpretation).

136 We next demonstrated the applicability of VIA in single-cell multi-omics analysis by inferring murine
137 *Isl1*+ cardiac progenitor cell (CPC) transition states using both single-cell transcriptomic and chromatin
138 accessibility information²⁰ (**Fig. 2d-i**). VIA consistently uncovers the bifurcating lineages towards the

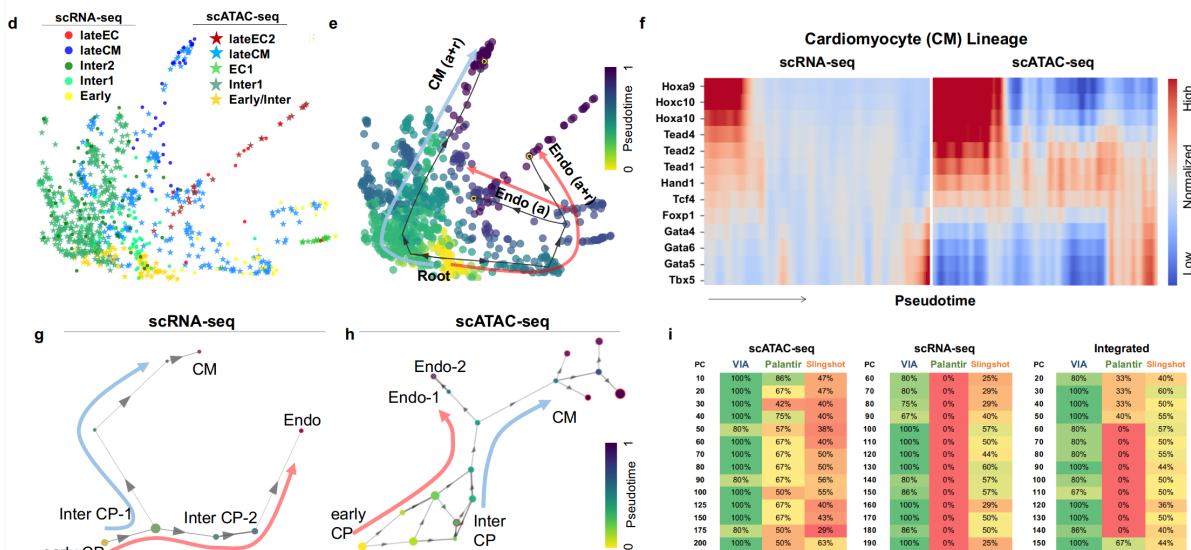
139 endothelial and cardiomyocyte fates based on the scRNA-seq, scATAC-seq datasets and their data
140 integration (see **Methods** for data integration). Other methods such as Palantir and Slingshot, that are also
141 applicable to non-transcriptomic data, fail to uncover the two main lineages in the individual as well as
142 the more challenging integrated multi-omic data. They typically only detect one of the two lineages and
143 instead falsely detect several intermediate and early stages as final cell fates (see **Fig. 2i** for prediction
144 accuracy). PAGA does not offer automated cell fate prediction and is therefore not benchmarked for this
145 dataset. VIA detects lineage pathways in both the scRNA-seq and scATAC-seq that can be used to
146 interpret relationships between transcription factor dynamics and gene expression in an unsupervised
147 manner. VIA automatically generates a pseudotemporal ordering of cells (without requiring manual
148 selection of relevant cells as done in Jia et al.²⁰) along respective lineages and their marker-TF pairs (see
149 **Fig. 2f and Supplementary Fig. S8e**). The highlighted gene and TF pairs in the cardiac lineage show a
150 strong correlation between expression and accessibility of *Gata* and Homeobox *Hox* genes which are
151 known to be related to the regulation of cardiomyocyte proliferation^{23,24,25}. VIA's reliable performance
152 against user-reconfiguration (choice of components, individual or integrated omic data) suggests it can be
153 used for transferable interpretation between scRNA-seq and chromatin accessibility data.

154 We further tested VIA on a wider scope of (small-to mid-sized) scRNA-seq datasets, including B-cell
155 differentiation²⁶, hematopoiesis^{2,27}, embryonic stem (ES) cell differentiation in embryoid bodies¹⁵, and
156 endocrine differentiation (~10² - 10⁴ cells). By comparing VIA with top-performing and popular TI
157 algorithms, e.g. PAGA²⁸, Palantir, SlingShot and CellRank¹³ (See **Methods**, and **Supplementary Fig.**
158 **S1-7** for full analysis), we showed that VIA consistently outperforms other methods in terms of both
159 runtime (in some cases by several magnitudes see **Supplementary Table S3 for runtime comparison**),
160 and more robust and accurate lineage prediction across a wide range of pre-processing and algorithmic
161 parameters. VIA's relaxation of graph traversal to permit cyclic sub-paths (see **Supplementary Fig. S1**)
162 and movements that are temporally reversed, augments its sensitivity to lineages. Notably, VIA more
163 consistently across a wide range of input parameter choice identified less populous lineages that were at
164 best detected by other methods for a narrow sweet spot of parameters. For example, VIA reliably
165 delineates the megakaryocyte, conventional and plasmacytoid dendritic cell (cDC and pDC) lineages in
166 human hematopoiesis (**Fig. 2m-o, Supplementary Fig. S3-4** for pseudotime and graph-topological gene
167 trends for all lineages); and Delta cells (3%) during the endocrine progenitor cells differentiation (**Fig.**
168 **2j-l, Supplementary Fig. S6** for pseudotime and topological gene trends for all lineages), as evidenced
169 by the corresponding gene-expression trend analysis and parameter stress tests. Interestingly, we find that
170 VIA often detects 2 Beta cell subpopulations (**Supplementary Fig. S6b,d,f**) that express typical Beta
171 markers like *Dkl1*, *Pdx1*, but differ in their expression of *Ins1* and *Ins2* (**Supplementary Fig. S6d**). Such
172 a Beta cell heterogeneity^{29,30}, whereby the immature Beta-2 population strongly expresses *Ins2*, and
173 weakly expresses *Ins1*, and the mature Beta-1 population expresses both types of *Ins*³⁰, can also be
174 reconciled based on the position of the Beta-2 cluster on the VIA graph (**Supplementary Fig. S6f**).

Large-scale (1.3 million cells) trajectory inference of mouse organogenesis

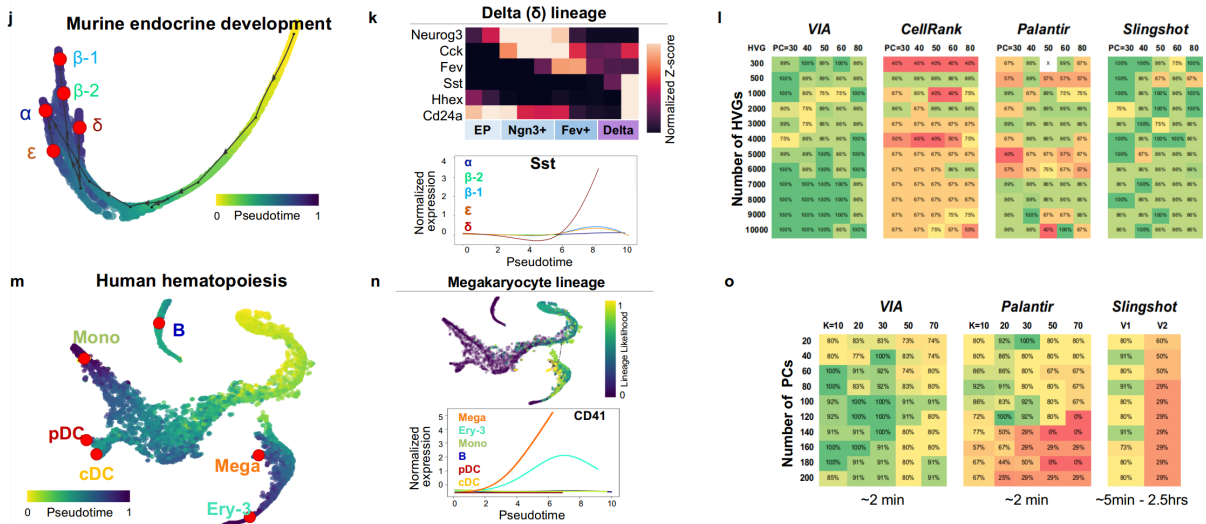


Multi-omic integration of cardiac progenitor cells (scRNA-seq + scATAC-seq)



175

Stress-testing of prediction of terminal states



176 **Figure 2 VIA accurately infers trajectories in diverse scRNA-seq datasets. (a)** VIA cluster-graph trajectory where
177 nodes are colored by pseudotime, and branches are shaded according to major lineages of 1.3-million-cell mouse
178 organogenesis cell atlas (MOCA). The VIA analysis (which is independent of the choice of visualization) produces a
179 connected structure with linkages between some of the major cell types that have a tendency to become segregated
180 in a UMAP based TI analysis (see **Supplementary Fig. S12-15**). The stem (root) branch consists of epithelial cells
181 derived from ectoderm and endoderm, leading to two main branches: 1) the mesenchymal and 2) the neural tube and
182 neural crest. Other major groups are placed in the biologically relevant neighborhoods, such as the adjacencies
183 between hepatocyte and epithelial trajectories; the neural crest (comprising glial cells and PNS neurons) and the
184 neural tube; as well as the links between early mesenchyme with both the hematopoietic cells and the endothelial
185 cells (see **Supplementary Note 3**). **(b)** (Left) Single-cell PHATE embedding colored by major cell groups. (Right)
186 Single-cell PHATE embedding colored by VIA pseudotime. **(c)** Lineage pathways and probabilities of neuronal,
187 myocyte and WBC lineages (see **Supplementary Fig. S6 for other lineages**). **(d)** scRNA-seq and scATAC-seq data
188 of *Isl1+* cardiac progenitors (CPs) integrated using Seurat3 before VIA TI analysis and PHATE visualization. Cells are
189 colored by annotated cell-type and experimental modality **(e)** Cells are colored by VIA pseudotime with the
190 VIA-inferred trajectory towards endothelial and myocyte lineages projected on top. **(f)** Marker gene expression and
191 chromatin accessibility for gene-TF pairs along pseudotime axis for Cardiomyocyte lineage **(g)** VIA-graph trajectory
192 with nodes colored by pseudotime shows bifurcation to endothelial and myocyte cells in scRNA-seq cells **(h)**
193 scATAC-seq of *Isl1+* CPs: VIA-graph again shows bifurcation after intermediate CP stage. **(i)** Lineage prediction
194 accuracy (F1-score) for methods that offer automated lineage detection and are not limited to transcriptomic data. **(k)**
195 Pancreatic Islets: Colored by VIA pseudotime with detected terminal states shown in red and annotated based on
196 known cell type as Alpha, Beta-1, Beta-2, Delta and Epsilon lineages where Beta-2 is *Ins1^{low}Ins2+* Beta subtype
197 (**Supplementary Fig. S7**). **(l)** VIA inferred cluster-level pathway shows gene regulation along endocrine progenitor
198 (EP) to Delta lineage specification (top) and *Sst* gene-expression trend shows rise of *Sst* in Delta lineage (See
199 **Supplementary Fig. S7 for remaining**). **(m)** Prediction Accuracy of the 4 major endocrine cell types when varying
200 the number of HVGs selected in pre-processing, and the number of PCs. **(n)** Human *CD34+* hematopoiesis with 6
201 detected cell fates annotated **(o)** lineage pathway and gene-pseudotime trend shown for the *CD41* Megakaryocytic
202 cells (see **Supplementary Fig. S3 for other lineages**). **(p)** Prediction accuracy of 6 cell fates when varying number
203 of K (nearest neighbors) and PCs. Note Slingshot on default mode ("V2") uses GMM clustering and "V1" uses
204 K-means clustering (allowing for over-clustering K=15, to increase sensitivity). Runtime of each method is also
205 highlighted below the chart.

206 VIA enables multi-omic analysis beyond transcriptomic data

207 Broad applicability of TI beyond transcriptomic analysis is increasingly critical, but existing methods
208 have limitations contending with the disparity in the data structure (e.g. sparsity and dimensionality)
209 across a variety of single-cell data types and oftentimes are designed with a view to only handling
210 transcriptomic data (e.g. methods using RNA velocity to infer directionality).

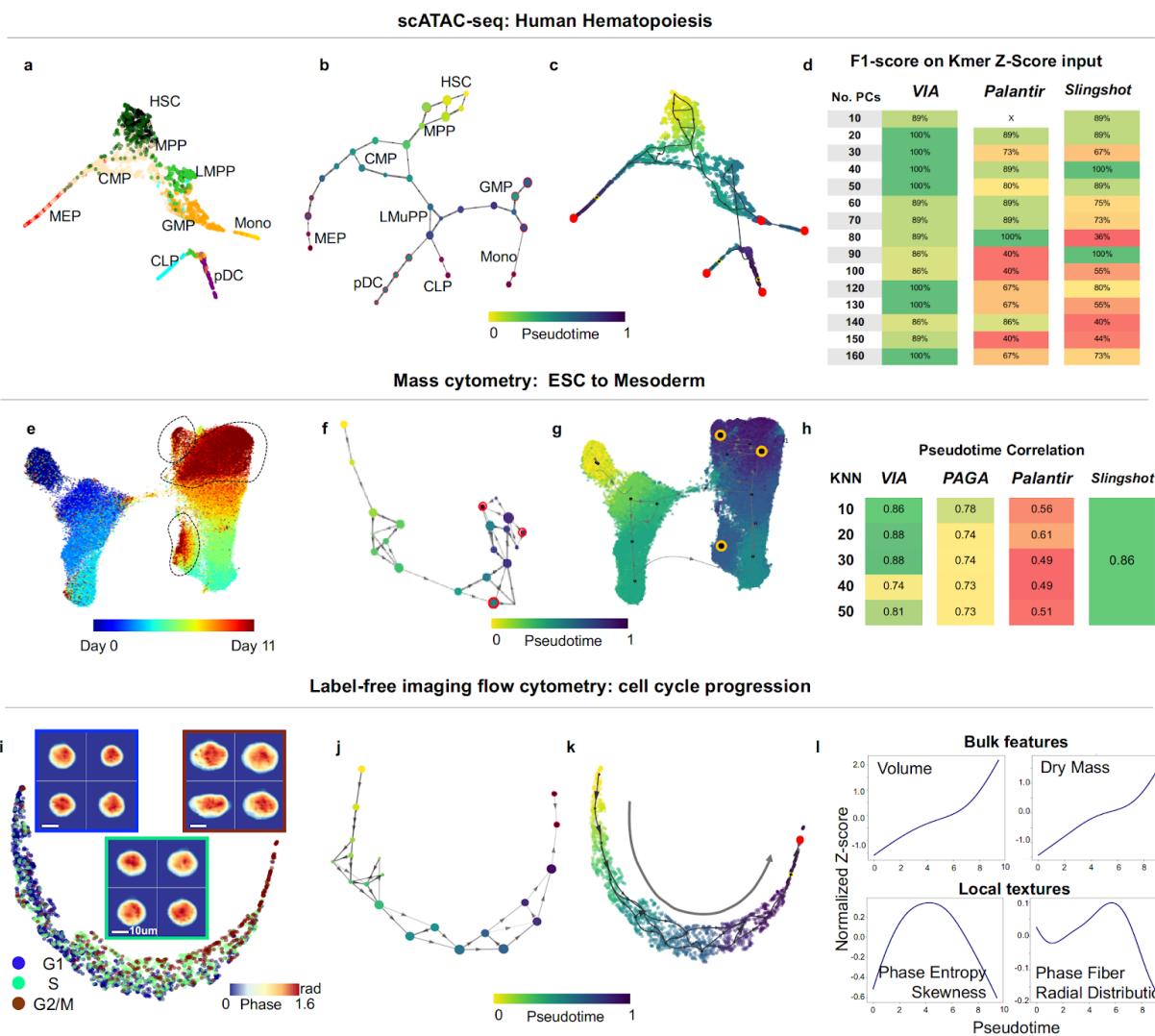
211 First, we employ VIA to analyze human scATAC-seq profiles (from *CD34+* human bone marrow) (**Fig.**
212 **3a**), and find that the continuous landscape of hematopoiesis generally mirrors the scRNA-seq human
213 hematopoietic data (**Fig. 2c**). The intrinsic sparsity of scATAC-seq data poses a challenge that can be
214 alleviated by choice of pre-processing pipelines, and we see that VIA consistently predicts the expected
215 hierarchy of furcations that leads to the lymphoid, myeloid and erythroid lineages for two commonly
216 accepted pre-processing protocols^{31,27} (**Methods**). This again holds across a wide range of input
217 parameters, as shown by the changes in the accessibility of TF motifs associated with known regulators,
218 e.g. *Gata1* (erythroid), *Cebpd* (myeloid) (**Fig 3b-d, Supplementary Fig. S7**).

219 We next investigated whether VIA can cope with a significant drop in data dimensionality (10-100), as
220 often presented in flow/mass cytometry data, and still delineate continuous biological processes. We run
221 VIA on a time-series mass cytometry data (28 antibodies, 90K cells) capturing murine embryonic stem
222 cells (ESCs) differentiation toward mesoderm cells (Day 0 - Day 11)³². Unlike previous analysis³² of the
223 same data which required chronological labels to visualize the developmental hierarchy, we ran VIA
224 without such supervised adjustments and accurately captured the sequential development. VIA computed
225 the trajectories with faster runtime (running in 2 minutes versus Slingshot which required 6 hours **see**
226 **Table S3**), detecting 3 terminal states corresponding to cells in the final developmental stages: 2
227 corresponding to the main region of Day 10-11 (marked by *Pdgfra*, *Cd44* and *Gata4* expressions), and a
228 small population of Day 10-11 cells expressing EpCAM, which are otherwise obscured in other methods
229 (e.g. Palantir, Slingshot), especially the small EpCAM population (~0.5% of cells) (**Fig 3e-h, Fig. S9e,f**).

230 Finally we tested the adaptability of VIA to infer cell-cycle stages based on label-free single-cell
231 biophysical morphology (38 features, **see Supplementary Table S4 and Table S5**) profiled by our
232 recently developed high-throughput imaging flow cytometer, called FACED³³. VIA reliably reconstructed
233 the continuous cell-cycle progressions from G1-S-G2/M phase of two different types of live breast cancer
234 cells as validated by the single-cell fluorescent (DNA dye) images captured by the same system
235 (**Methods**)(**Fig. 3i-k** for MCF7, **Supplementary Fig. S10** for MDA-MB231). Intriguingly, according to
236 the pseudotime ordered by VIA, not only can it reveal the known cell growth in size and mass³⁴, and
237 general conservation of cell mass density³⁵ (as derived from the FACED images (**Methods**)) throughout
238 the G1/S/G2 phases, but also a slow-down trend during the G1/S transition, consistent with the lower
239 protein-accumulation rate during S phase³⁶ (**Fig. 3l, Supplementary Fig. S10 f,g**). The variation in
240 biophysical textures (e.g. phase entropy) along the VIA pseudotime likely relates to known architectural
241 changes of chromosomes and cytoskeletons during the cell cycles (**Fig. 3l, Fig. S10 f,g**). These results
242 further substantiate the growing body of work^{37,38,39,40} on imaging biophysical cytometry for gaining a
243 mechanistic understanding of biological systems, especially when combined with omics analysis⁴¹.

244 Concluding Remarks

245 Overall, VIA offers an advancement to TI methods to study a diverse range of single-cell omic data,
246 including those targeted by many cell-atlas initiatives. By combining lazy-teleporting random walks and
247 MCMC simulations, VIA relaxes common constraints on graph traversal and causality. This enables
248 accurate lineage prediction that is robust to parameter configuration for a variety of complex topologies
249 and rarer lineages obscured in other methods. Our stress tests showed that the modeled developmental
250 landscape in other methods is vulnerable to user parameter choice which can incur fragmentation or
251 spurious linkages, and consequently only yield biologically sensible lineages for a narrow sweet spot of
252 parameters (See the summary in **Supplementary Fig. S16**). For example, due to algorithmic measures
253 taken to restrict permissible graph-edge transitions and progressively reduce the inherent dimensionality
254 (e.g. PCA followed by subsetting the number of diffusion components) other algorithms struggle to
255 delineate obscure lineages and maintain neighborhood relationships. VIA's wider bandwidth of accuracy,
256 superior runtime and preservation of global graph properties for very large datasets, offers a unique and
257 well-suited approach for multifaceted exploratory analysis to uncover novel biological processes,
258 potentially those deviated from healthy trajectories.



259 **Figure 3 VIA infers trajectories in single-cell multi-omic and image datasets** (a) Major lineages of human
 260 hematopoiesis (profiled by scATAC-seq) projected onto the UMAP embedding. Lineages are colored by FACS sorted
 261 labels²⁷. (b) VIA cluster-graph topology colored by VIA pseudotime. (c) Trajectory, pseudotime and detected terminal
 262 states (red) projected onto the UMAP embedding. (d) F1-scores (on the k-mer Z-score input) for terminal state
 263 prediction by different TI methods (for a fixed KNN = 20). Terminal states include megakaryocyte–erythroid progenitor
 264 (MEP), common lymphoid progenitor (CLP), plasmacytoid dendritic cell (pDC) and monocytes (Mono) lineages. The
 265 comparisons show that VIA's accuracy remains high across a wide range of PCs. (e) Differentiation of mESC to
 266 mesoderm cells measured by single-cell mass cytometry. UMAP embedding is colored by different measurement time
 267 points (Day 0-11). (f) VIA cluster graph with 3 detected terminal nodes (red) and colored by pseudotime. (g) VIA
 268 results projected onto single-cell UMAP embedding shows 3 terminal states correspond to Day 10/11 regions. (h)
 269 Correlation of inferred pseudotime and day-labels achieved by different TI methods. The benchmark was done across
 270 different numbers of KNN (using all 28 antibodies). (i) Label-free cell cycle progression tracking based on FACED
 271 imaging cytometry. The PHATE embedding is constructed using 38 biophysical/morphological features computed
 272 from images of human breast cancer cells (MCF7) (See **Supplementary Fig. S10** for additional results using another
 273 breast cancer cell type (MDA-MB231)). The embedding is colored by the known cell cycle stages given by the DNA
 274 fluorescence images (obtained from the same system). (j) VIA graph topology colored by pseudotime. (k) VIA
 275 trajectory and pseudotime projected on embedding. (l) “Biophysical” feature expressions (Z-score normalized) over
 276 pseudotime. (See **Supplementary Table S4-5** for detailed definitions of the features).

277 **Methods**

278 **VIA Algorithm**

279 VIA applies a scalable probabilistic method to infer cell state dynamics and differentiation hierarchies by
280 organizing cells into trajectories along a pseudotime axis in a nearest-neighbor graph which is the basis
281 for subsequent random walks. Single cells are represented by graph nodes that are connected based on
282 their feature similarity, e.g. gene expression, transcription factor accessibility motif, protein expression or
283 morphological features of cell images. A typical routine in VIA mainly consists of four steps:

284 1. **Accelerated and scalable cluster-graph construction.** VIA first represents the single-cell data in a
285 k-nearest-neighbor (KNN) graph where each node is a cluster of single cells. The clusters are
286 computed by our recently developed clustering algorithm, PARC¹¹. In brief, PARC is built on
287 hierarchical navigable small world (HNSW⁵⁸) accelerated KNN graph construction and a fast
288 community-detection algorithm (Leiden method⁴²), which is further refined by data-driven pruning.
289 The combination of these steps enables PARC to outperform other clustering algorithms in
290 computational run-time, scalability in data size and dimension (without relying on subsampling of
291 large-scale, high-dimensional single-cell data (>1 million cells)), and sensitivity of rare-cell detection.
292 We employ the cluster-level topology, instead of a single-cell-level graph, for TI as it provides a
293 coarser but clearer view of the key linkages and pathways of the underlying cell dynamics without
294 imposing constraints on the graph edges. Together with the strength of PARC in clustering scalability
295 and sensitivity, this step critically allows VIA to faithfully reveal complex topologies namely cyclic,
296 disconnected and multifurcating trajectories (**Supplementary Fig. S1**).

297 2. **Probabilistic pseudotime computation.** The trajectories are then modeled in VIA as (i)
298 lazy-teleporting random walk paths along which the pseudotime is computed and further refined by
299 (ii) MCMC simulations. The root is a single cell chosen by the user. These two sub-steps are detailed
300 as follows:

301 (i) *Lazy-teleporting random walk:* We first compute the pseudotime as the expected hitting time
302 of a *lazy-teleporting* random walk on an undirected cluster-graph generated in Step 1. The
303 lazy-teleporting nature of this random walk ensures that as the sample size grows, the expected
304 hitting time of each node does not converge to the stationary probability given by local node
305 properties, but instead continues to incorporate the wider global neighborhood information¹².
306 Here we highlight the derivation of the closed form expression of the hitting time of this modified
307 random walk with a detailed derivation in **Supplementary Note 2**.

308 The cluster graph constructed in VIA is mathematically defined as a weighted connected graph \mathbf{G}
309 (V, E, W) with a vertex set V of n vertices (or nodes), i.e. $V = \{v_1, \dots, v_n\}$ and an edge set E ,
310 i.e. a set of ordered pairs of distinct nodes. W is an $n \times n$ weight matrix that describes a set of
311 edge weights between node i and j , $w_{ij} \geq 0$ are assigned to the edges (v_i, v_j) . For an undirected
312 graph, $w_{ij} = w_{ji}$. The $n \times n$ probability transition matrix, P , of a standard random walk on this
313 graph \mathbf{G} can be given by

314
$$P = D^{-1}W \quad (1)$$

315 where D is the $n \times n$ degree matrix, which is a diagonal matrix of the weighted sum of the degree
316 of each node, i.e. the matrix elements are expressed as

$$d_{ij} = \begin{cases} \sum_k w_{ik} & , i = j \\ 0 & , i \neq j \end{cases} \quad (2)$$

317 where k are the neighbouring nodes connected to node i . Hence, d_{ii} (which can be reduced as d_i)
318 is the degree of node i . We next consider a *lazy* random walk, defined as Z , with probability
319 $(1 - x)$ of being lazy (where $0 < x < 1$), i.e. staying at the same node, then

320
$$Z = xP + (1 - x)I \quad (3)$$

321 where I is the identity matrix. When teleportation occurs with a probability $(1 - \alpha)$, the modified
322 lazy-teleporting random walk Z' can be written as follows, where J is an $n \times n$ matrix of ones.

323
$$Z' = \alpha Z + (1 - \alpha) \frac{1}{n} J \quad (4)$$

324 Here we adapt the concept of personalized PageRank vector, originally used for recording (or
325 *ranking*) personal preferences of a web-surfer toward particular website pages⁴³, to *rank* the
326 importance of other nodes (clusters of cells) to a given node, depending on the similarities among
327 nodes (related to P in the graph), and the lazy-teleporting random walk characteristics in the
328 graph (set by probabilities of teleporting and being lazy). Based on this concept, one could model
329 the likelihood to transit from one node (cluster of cells) to another, and thus construct the
330 pseudotime based on the hitting time, which is a parameter describing the expected number of
331 steps it takes for a random walk that starts at node i and visit node j for the first time. Consider
332 the teleporting probability of $(1 - \alpha)$ and a seed vector s specifying the initial probability
333 distribution across the n nodes (such that $\sum_m s_m = 1$, where s_m is the probability of starting at
334 node m) the personalized PageRank vector $pr_\alpha(s)$ (which is defined as a column vector) is the
335 unique solution to⁵⁶

336
$$pr_\alpha(s)^T = \alpha pr_\alpha(s)^T Z + (1 - \alpha) s^T. \quad (5)$$

337 Substituting Z (Eq. (3)) into Eq. (5), we can express the personalized PageRank vector $pr_\alpha(s)$ in
338 terms of the inverse of the β -normalized Laplacian, $R_{\beta,NL}$ of the modified random walk
339 (**Supplementary Note 2**), i.e.

340
$$pr_\alpha(s)^T = \beta s^T D^{-0.5} R_{\beta,NL} D^{0.5}, \quad (6)$$

341 where $\beta = \frac{2(1-\alpha)}{(2-\alpha)}$, and $R_{\beta,NL} = \sum_{m=1} \frac{\Phi_m \Phi_m^T}{[\beta + 2x(1-\beta)\eta_m]}$. Φ_m and η_m are the m^{th} eigenvector and
342 eigenvalue of the normalized Laplacian. In the expression of $R_{\beta,NL}$, the β and x regulate the
343 weight of contribution in each eigenvalue-eigenvector pair of the summation such that the first
344 eigenvalue-eigenvector pair (corresponding to the stationary distribution and given by the
345 local-node degree-properties) remains included in the overall expression, but does not overwhelm
346 the global information provided by subsequent ‘eigen-pairs’. Moreover, computation of $R_{\beta,NL}$ is
347 not limited to a subset of the first k eigenvectors (bypassing the need for the user to select a
348 suitable threshold or subset of eigenvectors) since the dimensionality is not on the order of

349 number of cells, but equal to the number of clusters and hence all eigenvalue-eigenvector pairs
 350 can be incorporated without causing a bottleneck in runtime.

351 The expected hitting time from node q to node r is given by⁴⁴,

$$352 \quad h_{\alpha}(q, r) = \frac{[pr_{\alpha}(e_r)^T](r)}{d_r} - \frac{[pr_{\alpha}(e_r)^T](q)}{d_q} \quad (7)$$

353 where e_i is an indicator vector with 1 in the i^{th} entry and 0 elsewhere (i.e. $s_m = 1$ if $m = i$ and
 354 $s_m = 0$ if $m \neq i$). We can substitute Eq. (6) into Eq. (7), making use of the fact that
 355 $\frac{1}{d_r} = [D^{-1}e_r]^T(r)$, and $D^{-0.5}R_{\beta,NL}D^{-0.5}$ is symmetric, to obtain a closed form expression of the
 356 hitting time in terms of $R_{\beta,NL}$

$$357 \quad h_{\alpha}(q, r) = \beta(e_r - e_q)^T D^{-0.5}R_{\beta,NL}D^{-0.5}e_r \quad (8)$$

358 (ii) *MCMC simulation*: The hitting time metric computed in Step-1 is used to infer
 359 graph-directionality. Instead of pruning edges in the ‘reverse’ direction, edge-weights are biased
 360 based on the time difference between nodes using the logistic function with growth factor $b=1$.

$$361 \quad f(t) = \frac{1}{1+e^{-b(t_1-t_0)}}$$

362 We then recompute the pseudotimes on the forward biased graph: Since there is no closed form
 363 solution of hitting times on a *directed* graph, we perform MCMC simulations (paralelly processed
 364 to enable fast simulations of 1000s of teleporting, lazy random walks starting at the root node of
 365 the cluster graph) and use the first quartile of the simulated pseudotime values for a respective
 366 node as the refined pseudotime for that node relative to the root. This refinement step ensures that
 367 the pseudotime is robust to the spurious links (or conversely, links that are too weakly weighted)
 368 that can distort calculations based purely on the closed form solution of hitting times
 369 (**Supplementary Fig. S9d**). By using this 2-step pseudotime computation, VIA mitigates the
 370 issues of convergence issues and spurious edge-weights, both of which are common in
 371 random-walk pseudotime computation on large and complex datasets¹².

372 3. **Automated terminal-state detection.** The algorithm then uses the refined directed and weighted
 373 graph (the edges are re-weighted using the refined pseudotimes) to predict which nodes represent the
 374 terminal states based on a consensus vote of pseudotime and multiple vertex connectivity properties,
 375 including out-degree (i.e. the number of edges directing out from the node), closeness $C(q)$, and
 376 betweenness $B(q)$.

$$377 \quad C(q) = \frac{1}{\sum_{q \neq r} l(q,r)}$$

$$378 \quad B(q) = \sum_{r \neq q \neq t} \frac{\sigma_{rt}(q)}{\sigma_{rt}}$$

379 $l(q, r)$ is the distance between node q and node r (i.e. the sum of edges in a shortest path connecting
 380 them). σ_{rt} is the total number of shortest paths from node r to node t . $\sigma_{rt}(q)$ is the number of these

381 paths passing through node q . The consensus vote is performed on nodes that score above (or below
382 for out-degree) the median in terms of connectivity properties. We show on multiple simulated and
383 real biological datasets that VIA more accurately predicts the terminal states across a range of input
384 data dimensions and key algorithm parameters than other methods attempting the same
385 (**Supplementary Fig. S16**).

386 4. **Automated trajectory reconstruction.** VIA then identifies the most likely path of each lineage by
387 computing the likelihood of a node traversing towards a particular terminal state (e.g. differentiation).
388 These lineage likelihoods are computed as the visitation frequency under lazy-teleporting MCMC
389 simulations from the root to a particular terminal state, i.e. the probability of *node i* reaching
390 *terminal-state j* as the number of times *cell i* is visited along a successful path (i.e. *terminal-state j* is
391 reached) divided by the number of times *cell i* is visited along all of the simulations. In contrast to
392 other trajectory reconstruction methods which compute the shortest paths between root and terminal
393 node^{1,2}, the lazy-teleporting MCMC simulations in VIA offer a probabilistic view of pathways under
394 relaxed conditions that are not only restricted to the random-walk along a tree-like graph, but can also
395 be generalizable to other types of topologies, such as cyclic or connected/disconnected paths. In the
396 same vein, we avoid confining the graph to an absorbing Markov chain^{13,3} (AMC) as this places
397 prematurely strict / potentially inaccurate constraints on node-to-node mobility and can impede
398 sensitivity to cell fates (as demonstrated by VIA's superior cell fate detection across numerous
399 datasets (**Supplementary Fig. S16**)).

400 **Downstream visualization and analysis**

401 VIA generates a visualization that combines the network topology and single-cell level
402 pseudotime/lineage probability properties onto an embedding based on UMAP or PHATE. Generalized
403 additive models (GAMs) are used to draw edges found in the high-dimensional graph onto the lower
404 dimensional visualization (**Fig. 1**). An unsupervised downstream analysis of cell features (e.g. marker
405 gene expression, protein expression or image phenotype) along pseudotime for each lineage is performed
406 (**Fig. 1**). Specifically, VIA plots the expression of features across pseudotime for each lineage by using
407 the lineage likelihood properties to weight the GAMs. A cluster-level lineage pathway is automatically
408 produced by VIA to visualize feature heat maps at the cluster-level along a lineage-path to see the
409 regulation of genes. VIA provides the option of gene imputation before plotting the lineage specific gene
410 trends. The imputation is fast as it relies on the single-cell KNN (scKNN) graph computed in Step 1.
411 Using an affinity-based imputation method⁴⁵, this step computes a “diffused” transition matrix on the
412 scKNN graph used to impute and denoise the original gene expressions.

413 **Benchmarked Methods**

414 The methods were mainly chosen based on their superior performance in a recent large-scale
415 benchmarking study⁴, including a select few recent methods claiming to supersede those in the study.
416 Specifically, recent and popular methods exhibiting reasonable scalability, and automated cell fate
417 prediction in multi-lineage trajectories were favoured as candidates for benchmarking (See
418 **Supplementary Table S1** for the key characteristics of methods). Performance stress-tests in terms of
419 lineage detection of each biological dataset, and pseudotime correlation for time-series data were

420 conducted over a range of key input parameters (e.g. numbers of k-nearest neighbors, highly variable
421 genes (HVGs), and principal components (PCs)) and pre-processing protocols (see **Fig. 2m,p**,
422 **Supplementary Fig. 16**). All comparisons were run on a computer with an Intel(R) Xeon (R) W-2123
423 central processing unit (3.60GHz, 8 cores) and 126 GB RAM.

424 Quantifying terminal state prediction accuracy for parameter tests was done using the F1-score, defined
425 as the harmonic mean of recall and precision and calculated as:

$$426 \quad F_1 = \frac{tp}{tp + 0.5(fp + fn)}$$

427 Where tp is a true-positive: the identification of a terminal cluster that is in fact a final differentiated cell
428 fate; fp is a false positive identification of a cluster as terminal when in fact it represents an intermediate
429 state; and fn is a false negative where a known cell fate fails to be identified

430 **PAGA**²⁸. It uses a cluster-graph representation to capture the underlying topology. PAGA computes a
431 unified pseudotime by averaging the single-cell level diffusion pseudotime computed by DPT, but
432 requires manual specification of terminal cell fates and clusters that contribute to lineages of interest in
433 order to compare gene expression trends across lineages.

434 **Palantir**². It uses diffusion-map⁴⁶ components to represent the underlying trajectory. Pseudotimes are
435 computed as the shortest path along a KNN-graph constructed in a low-dimensional diffusion component
436 space, with edges weighted such that the distance between nodes corresponds to the diffusion
437 pseudotime⁴⁷. (DPT). Terminal states are identified as extrema of the diffusion maps that are also outliers
438 of the stationary distribution. The lineage-likelihood probabilities are computed using Absorbing Markov
439 Chains (constructed by removing outgoing edges of terminal states, and thresholding reverse edges).

440 **Slingshot**¹. It is designed to process low-dimensional embeddings of the single-cell data. By default
441 Slingshot runs clustering based on Gaussian mixture modeling and recommends using the first few PCs as
442 input. Slingshot connects the clusters using a minimum spanning tree and then fits principle curves for
443 each detected branch. It uses the orthogonal projection against each principal curve to fit a separate
444 pseudotime for each lineage, and hence the gene expressions cannot be compared across lineages. Also,
445 the runtimes are prohibitively long for large datasets or high input dimensions.

446 **CellRank**¹³. This method combines the information of RNA velocity (computed using scVelo⁴⁸) and
447 gene-expression to infer trajectories. Given it is mainly suited for the scRNA-seq data, with the
448 RNA-velocity computation limiting the overall runtime for larger dataset, we limit our comparison to the
449 pancreatic dataset which the authors of CellRank used to highlight its performance.

450 **Simulated Data**

451 We employed the DynToy⁴ (<https://github.com/dynverse/dyntoy>) package, which generates synthetic
452 single-cell gene expression data (~1000 cells x 1000 'genes'), to simulate different complex trajectory
453 models. Using these datasets, we tested that VIA consistently and more accurately captures both tree and
454 non-tree like structures (multifurcating, cyclic, and disconnected) compared to other methods

455 **(Supplementary Fig. S1)**. All methods are subject to the same data pre-processing steps, PCA dimension
456 reduction and root-cell to initialize the path.

457 **Multifurcating structure**. This dataset consists of 1000 ‘cells’ multifurcating into 4 terminal states. VIA
458 robustly captures all four terminal cell fates across a range of input PCs and the pseudotimes are well
459 inferred relative to the root node (**Supplementary Fig. S1a**). Note that two terminal states (M2 and M8),
460 which are very close to each other, are easily merged by the other methods (Slingshot, Palantir and
461 PAGA).

462 **Cyclic structure**. We ran VIA and other methods for different values of K nearest neighbors. VIA
463 unambiguously shows a cyclic network for a range of K (in KNN). Slingshot does not use a KNN
464 parameter and shows 3 fragmented different lineages (top to bottom). PAGA fails to capture the
465 connected cyclic structure at K = 10 and 5, while Palantir visually shows a linear (K = 10, 30) or
466 disconnected structure (K = 5). Van den Berge et al⁵⁷ note that the challenge of cyclic trajectory
467 reconstruction is also common in other popular methods, such as Monocle3 that consistently fragments or
468 fits branching structures onto cyclic simulated datasets.

469 **Disconnected structure**. This dataset comprises two disconnected trajectories (T1 and T2). T1 is cyclic
470 with an extra branch (M5 to M6), T2 has a bifurcation at M3 (**Supplementary Fig. S1c**). VIA captures
471 the two disconnected structures as well as the M6 branch in the cyclic structure, and the bifurcation in the
472 smaller structure. PAGA captures the underlying structure at PC = 20 but becomes fragmented for other
473 numbers of PCs. Palantir also yields multiple fragments and is not able to capture the overall structure,
474 while Slingshot (using the default clustering based on Gaussian mixture modeling) connects T1 and T2,
475 and only captures one of the bifurcations in T1.

476 **Biological Data**

477 The pre-processing steps described below for each dataset are not included in the reported runtimes as
478 these steps are typically very fast, (typically less than 1-10% of the total runtime depending on the
479 method. E.g. only a few minutes for pre-processing 100,000s of cells) and only need to be performed
480 once as they remain the same for all subsequent analyses. It should also be noted that visualization (e.g.
481 UMAP, t-SNE) are not included in the runtimes. VIA provides a subsampling option at the visualization
482 stage to accelerate this process for large datasets without impacting the previous computational steps.
483 However, to ensure fair comparisons between TI methods (e.g. other methods do not have an option to
484 compute the embedding on a subsampled input and transfer the results between the full trajectory and the
485 sampled visualization, or rely on a slow version of tSNE), we simply provide each TI method with a
486 pre-computed visualization embedding on which the computed results are projected.

487 **ScRNA-seq of mouse pre-B cells**. This dataset²⁶ models the pre-BI cell (Hardy fraction C’) process
488 during which cells progress to the pre-BII stage and B cell progenitors undergo growth arrest and
489 differentiation. Measurements were obtained at 0, 2, 6, 12, 18 and 24 hours (h) for a total of 313 cells x
490 9,075 genes. We follow a standard Scanpy preprocessing recipe⁴⁹ that filters cells with low counts, and
491 genes that occur in less than 3 cells. The filtered cells are normalized by library size and log transformed.
492 The top 5000 highly variable genes (HVG) are retained. Cells are renormalized by library count and
493 scaled to unit variance and zero mean. VIA identifies the terminal state at 18-24 h and accurately

494 recapitulates the gene expression trends²⁶ along inferred pseudotime of *IgIII*, *Slc7a5*, *Foxo1*, *Myc*, *Ldha*
495 and *Lig4*. (**Supplementary Fig. S2a**). We show the results generalize across a range of PCs for two
496 values of K of the graph with higher accuracy in locating the later cell fates than Slingshot and Palantir.
497 (**Supplementary Fig. S2b**).

498 **ScRNA-seq of human CD34+ bone marrow cells.** This is a scRNA-seq dataset of 5800 cells
499 representing human hematopoiesis². We used the filtered, normalized and log-transformed count matrix
500 provided by Setty et al²., with PCA performed on all the remaining genes. The cells were annotated using
501 SingleR⁵⁰ which automatically labeled cells based on the hematopoietic reference dataset Novershtern
502 Hematopoietic Cell Data - GSE24759⁵¹. The annotations are in agreement with the labels inferred by
503 Setty et al. for the 7 clusters, including the root HSCs cluster that differentiates into 6 different lineages:
504 monocytes, erythrocytes, and B cells, as well as the less populous megakaryocytes, cDCs and pDCs. VIA
505 consistently identifies these lineages across a wider range of input parameters and data dimensions (e.g.
506 the number of K and PCs provided as input to the algorithms see **Fig. 2p**, and **Supplementary Fig. S3c**).
507 Notably, the upregulated gene expression trends of the small populations can be recovered in VIA, i.e.
508 pDC and cDC show elevated CD123 and CSF1R levels relative to other lineages, and the upregulated
509 CD41 expression in megakaryocytes (**Supplementary Fig. S3-S4**).

510 **ScRNA-seq of human embryoid body.** This is a mid-sized scRNA-seq dataset of 16,825 human cells in
511 embryoid bodies (EBs)¹⁵. We followed the same pre-processing steps as Moon et al. to filter out dead
512 cells and those with too high or low library count. Cells are normalized by library count followed by
513 square root transform. Finally the transformed counts are scaled to unit variance and zero mean. The
514 filtered data contained 16825 cells \times 17580 genes. PCA is performed on the processed data before
515 running each TI method. VIA identifies 6 cell fates, which, based on the upregulation of marker genes as
516 cells proceed towards respective lineages, are in accord with the annotations given by Moon et al., (See
517 the gene heatmap and changes in gene expression along respective lineage trajectories in **Supplementary**
518 **Fig. S5**). Note that Palantir and Slingshot do not capture the cardiac cell fate, and Slingshot also misses
519 the neural crest (see the F1-scores summary for terminal state detection **Supplementary Fig. S5**).

520 **ScRNA-seq of mouse organogenesis cell atlas.** This is a large and complex scRNA-seq dataset of mouse
521 organogenesis cell atlas (MOCA) consisting of 1.3 million cells⁶. The dataset contains cells from 61
522 embryos spanning 5 developmental stages from early organogenesis (E9.5-E10.5) to organogenesis
523 (E13.5). Of the 2 million cells profiled, 1.3 million are ‘high-quality’ cells that are analysed by VIA. The
524 runtime is approximately 40 minutes which is in stark contrast to the next fastest tool Palantir which takes
525 4 hours (excluding visualization). The authors of MOCA manually annotated 38 cell-types based on the
526 differentially expressed genes of the clusters. In general, each cell type exclusively falls under one of 10
527 major and disjoint trajectories inferred by applying Monocle3 to the UMAP of MOCA. The authors
528 attributed the disconnected nature of the 10 trajectories to the paucity of earlier stage common
529 predecessor cells. We followed the same steps as Cao et al.⁶ to retain high-quality cells (i.e. remove cells
530 with less than 400 mRNA, and remove doublet cells and cells from doubled derived sub-clusters). PCA
531 was applied to the top 2000 HVGs with the top 30 PCs selected for analysis. VIA analyzed the data in the
532 high-dimensional PC space. We bypass the step in Monocle3⁶ which applies UMAP on the PCs prior to
533 TI as this incurs an additional bias from choice of manifold-learning parameters and a further loss in

534 neighborhood information. As a result, VIA produces a more connected structure with linkages between
535 some of the major cell types that become segregated in UMAP (and hence Monocle3), and favors a
536 biologically relevant interpretation (**Fig. 2, Supplementary Fig. S11**). A detailed explanation of these
537 connections (graph-edges) extending between certain major groups using references to literature on
538 organogenesis is presented in **Supplementary Note 3**.

539 **ScRNA-seq of murine endocrine development**⁵. This is an scRNA-seq dataset of E15.5 murine
540 pancreatic cells spanning all developmental stages from an initial endocrine progenitor-precursor (EP)
541 state (low level of *Ngn3*, or *Ngn3*^{low}), to the intermediate EP (high level of *Ngn3*, or *Ngn3*^{high}) and *Fev*⁺
542 states, to the terminal states of hormone-producing alpha, beta, epsilon and delta cells^{5,5}. Following steps
543 by Lange et al¹³, we preprocessed the data using scVelo to filter genes, normalize each cell by total counts
544 over all genes, keep the top most variable genes, and take the log-transform. PCA was applied to the
545 processed gene matrix. We assessed the performance of VIA and other TI methods (CellRank, Palantir,
546 Slingshot) across a range of number of retained HVGs and input PCs (**Fig. 2m, Supplementary Fig. S6**).

547 **scATAC-seq of human bone marrow cells**. This scATAC-seq data profiles 3072 cells isolated from
548 human bone marrow using fluorescence activated cell sorting (FACS), yielding 9 populations²⁷: HSC,
549 MPP, CMP, CLP, LMPP, GMP, MEP, mono and plasmacytoid DCs (**Fig. 3a and Supplementary Fig.**
550 **S7**). We examined TI results for two different preprocessing pipelines to gauge how robust VIA is on the
551 scATAC-seq analysis which is known to be challenging for its extreme intrinsic sparsity. We used the
552 pre-processed data consisting of PCA applied to the z-scores of the transcription factor (TF) motifs used
553 by Buenrostro et al²⁷. Their approach corrects for batch effects in select populations and weighting of PCs
554 based on reference populations and hence involves manual curation. We also employed a more general
555 approach used by Chen et al.³¹ which employs ChromVAR to compute k-mer accessibility z-scores across
556 cells. VIA infers the correct trajectories and the terminal cell fates for both of these inputs, again across a
557 wide range of input parameters (**Fig. 3d and Supplementary Fig. S7**).

558 **ScRNA-seq and scATAC-seq of *Isl1*+ cardiac progenitor cells**. This time-series dataset captures
559 murine *Isl1*+ cardiac progenitor cells (CPCs) from E7.5 to E9.5 characterized by scRNA-seq (197 cells)
560 and scATAC-seq (695 cells)²⁰. The *Isl1*+ CPCs are known to undergo multipotent differentiation to
561 cardiomyocytes or endothelial cells. For the scRNA-seq data, the quality filtered genes and the size-factor
562 normalized expression values are provided by Jia et al.²⁰ as a “Single Cell Expression Set” object in R.
563 Similarly, the cells in the scATAC-seq experiment were provided in a “SingleCellExperiment” object with
564 low quality cells excluded from further analysis. The accessibility of peaks was transformed to a binary
565 representation as input for TF-IDF (term frequency-inverse document frequency) weighting prior to
566 singular value decomposition (SVD). The highlighted TF motifs in the heatmap (**Fig. 2j**) correspond to
567 those highlighted by Jia et al. We tested the performance when varying the number of SVDs used. We
568 also considered the outcome when merging the scATAC-seq and scRNA-seq data using Seurat3⁵².
569 Despite the relatively low cell count of both datasets, and the relatively under-represented scRNA-seq cell
570 count, the two datasets overlapped reasonably well and allowed us to infer the expected lineages in an
571 unsupervised manner (**Fig. 2d and Supplementary Fig. S8**). In contrast, Jia et al., performed a supervised
572 TI by manually selecting cells relevant to the different lineages (for the scATAC-seq cells) and choosing
573 the two diffusion components that best characterize the developmental trajectories in low dimension²⁰.

574 **Mass cytometry data of mouse embryonic stem cells (mESC).** This is a mass cytometry (or CyTOF)
575 dataset, consisting of 90,000 cells and 28 antibodies (corresponding to ~7000 cells each from Day 0-11
576 measurements), that represents differentiation of mESC to mesoderm cells³². An arcsinh transform with a
577 scaling factor of 5 was applied on all features - a standard procedure for CyTOF datasets, followed by
578 normalization to unit variance and zero mean. Given the small feature set, no PCA is required
579 **(Supplementary Fig. S9).** VIA identifies 3 main terminal states corresponding to Day 11 and Day 10,
580 Palantir on the other hand identifies three Terminal states that all correspond to Days in the first half of
581 the experiment and the pseudotime is heavily influenced by the root node being very weakly connected to
582 the other stages of the process. Slingshot appears to capture the overall pseudotime but the 6 lineages
583 imposed onto the low dimensional representation are difficult to interpret and distinguish. To improve
584 Palantir performance we used 5000 waypoints but this takes almost 20 minutes to complete (excluding
585 time taken for embedding the visualization). VIA runs in ~3 minutes and produces results consistent with
586 the known ordering. The pseudotime reflects the range of Days very well, even capturing the small
587 population of Day 11 cells on the left hand side of the Day 6 cells in the embedding **(Fig. 3, and**
588 **Supplementary Fig. S9).**

589 **Single-cell biophysical phenotypes derived from imaging flow cytometry.** This is the in-house dataset
590 of single-cell biophysical phenotypes of two different human breast cancer types (MDA-MB231 and
591 MCF7). Following our recent image-based biophysical phenotyping strategy^{53,54}, we defined the
592 spatially-resolved biophysical features of a cell in a hierarchical manner based on both bright-field and
593 quantitative phase images captured by the FACED imaging flow cytometer (i.e., from the bulk features to
594 the subcellular textures). At the bulk level, we extracted the cell size, dry mass density, and cell shape. At
595 the subcellular texture level, we parameterized the global and local textural characteristics of optical
596 density and mass density at both the coarse and fine scales (e.g., local variation of mass density, its
597 higher-order statistics, phase entropy radial distribution etc.). This hierarchical phenotyping approach^{53,54}
598 allowed us to establish a single-cell biophysical profile of 38 features, which were normalized based on
599 the z-score **(See Supplementary Table S4 and Table S5).** All these features, without any PCA, are used
600 as input to VIA. In order to weigh the features, we use a mutual information classifier to rank the features,
601 based on the integrated fluorescence intensity of the fluorescence FACED images of the cells (which
602 serve as the ground truth of the cell-cycle stages). Following normalization, the top 3 features (which
603 relate to cell size) are weighted (using a factor between 3-10).

604 **Imaging flow cytometry experiment**

605 **FACED imaging flow cytometer setup**

606 A multimodal FACED imaging flow cytometry (IFC) platform was used to obtain the quantitative phase
607 and fluorescence images of single cells in microfluidic flow at an imaging throughput of $\sim 70,000$
608 cells/sec. The light source consisted of an Nd:YVO picosecond laser (center wavelength = 1064 nm,
609 Time-Bandwidth) and a periodically-poled lithium niobate (PPLN) crystal (Covesion) for second
610 harmonic generation of a green pulsed beam (center wavelength = 532 nm) with a repetition rate of 20
611 MHz. The beam was then directed to the FACED module, which mainly consists of a pair of
612 almost-parallel plane mirrors. This module generated a linear array of 50 beamlets (foci) which were
613 projected by an objective lens (40X, 0.6NA, MRH08430, Nikon) on the flowing cells in the microfluidic
614 channel for imaging. Each beamlet was designed to have a time delay of 1 ns with the neighboring
615 beamlet in order to minimize the fluorescence crosstalk due to the fluorescence decay. Detailed
616 configuration of the FACED module can be referred to Wu et al.³³. The epi-fluorescence image signal
617 was collected by the same objective lens and directed through a band-pass dichroic beamsplitter (center:
618 575nm, bandwidth: 15nm). The filtered orange fluorescence signal was collected by the photomultiplier
619 tube (PMT) (rise time: 0.57 ns, Hamamatsu). On the other hand, the transmitted light through the cell was
620 collected by another objective lens (40X, 0.8NA, MRD07420, Nikon). The light was then split equally by
621 the 50:50 beamsplitter into two paths, each of which encodes different phase-gradient image contrasts of
622 the same cell (a concept similar to Scherlien photography⁵⁵). The two beams are combined,
623 time-interleaved, and directed to the photodetector (PD) (bandwidth: >10 GHz, Alphas) for detection.
624 The signals obtained from both PMT and PD were then passed to a real-time high-bandwidth digitizer (20
625 GHz, 80 GS/s, Lecroy) for data recording.

626 **Cell culture and preparation**

627 MDA-MB231 (ATCC) and MCF7 (ATCC), which are two different breast cancer cell lines, were used for
628 the cell cycle study. The culture medium for MDA-MB231 was ATCC modified RPMI 1640 (Gibco)
629 supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% antibiotic-antimycotic (Anti-Anti)
630 (Gibco), while that for MCF7 was DMEM supplemented with 10% FBS (Gibco) and 1% Anti-Anti
631 (Gibco). The cells were cultured inside an incubator under 5% CO₂ and 37°C, and subcultured twice a
632 week. 1e6 cells were pipetted out from each cell line and stained with Vybrant DyeCycle orange stain
633 (Invitrogen).

634 Data Availability

635 Data used in Figures 1-3 as well as Supplementary Figures S1-S15) is available on:

- 636 1. Pancreatic data: Gene Expression Omnibus (GEO) under accession code GSE132188.
- 637 2. Cardiac progenitor data is available from the ENA repository under the accession code
638 PRJEB23303 or from [<https://github.com/loosolab/cardiac-progenitors>].
- 639 3. B-cell: STATegraData GitHub repository. [<https://github.com/STATegraData/STATegraData>]
- 640 4. Mass cytometry mesoderm: Cytobank
641 [<https://community.cytobank.org/cytobank/experiments/71953>].
- 642 5. Raw and processed data for scRNA-seq Human Hematopoiesis are available through the Human
643 Cell Atlas data portal at
644 <https://data.humancellatlas.org/explore/projects/091cf39b-01bc-42e5-9437-f419a66c8a45>.
- 645 6. Embryoid Body: Mendeley Data repository at <https://doi.org/10.17632/v6n743h5ng.1>.
- 646 7. Mouse Organogenesis : NCBI Gene Expression Omnibus under accession number GSE119945
- 647 8. FACED cell cycle: <https://github.com/ShobiStassen/VIA> and on FigShare
648 <https://doi.org/10.6084/m9.figshare.13601405.v1>
- 649 9. scATAC-seq Hematopoiesis: GEO: GSE96772. Processed scATAC-seq data, which include PC
650 values and TF scores per cell can be found in Data S1. of
651 <https://doi.org/10.1016/j.cell.2018.03.074>
- 652 10. Toy Data: <https://github.com/ShobiStassen/VIA>

653 Code Availability

654 VIA is available as a pip installable python library “pyVIA” with tutorials and sample data available on
655 <https://github.com/ShobiStassen/VIA> and <https://pypi.org/project/pyVIA/>

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