Determination of essential phenotypic elements of clusters in high-dimensional entities - DEPECHE

Short title: DEPECHE-a data-mining algorithm for mega-variate data

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Abstract

Technological advances have facilitated an exponential increase in the amount of information that can be derived from single cells, necessitating new computational tools that can make this highly complex data interpretable. Here, we introduce DEPECHE, a rapid, parameter free, sparse k-means-based algorithm for clustering of multi- and megavariate single-cell data. In a number of computational benchmarks aimed at evaluating the capacity to form biologically relevant clusters, including flow/mass-cytometry and single cell RNA sequencing data sets with manually curated gold standard solutions, DEPECHE clusters as well or better as the best performing state-of-the-art clustering algorithms. However, the main advantage of DEPECHE, compared to the state-of-the-art, is its unique ability to enhance interpretability of the formed clusters, in that it only retains variables relevant for cluster separation, thereby facilitating computational efficient analyses as well as understanding of complex datasets. An open source R implementation of DEPECHE is available at https://github.com/theorell/DepecheR.

Author summary

DEPECHE-a data-mining algorithm for mega-variate data

Modern experimental technologies facilitate an array of single cells measurements, *e.g.* at the RNA-level, generating enormous datasets with thousands of annotated biological markers for each of thousands of cells. To analyze such datasets, researchers routinely apply automated or semi-automated techniques to order the cells

into biologically relevant groups. However, even after such groups have been generated, it is often difficult to interpret the biological meaning of these groups since the definition of each group often dependends on thousands of biological markers. Therefore, in this article, we introduce DEPECHE, an algorithm designed to simultaneously group cells and enhance interpretability of the formed groups. DEPECHE defines groups only with respect to biological markers that contribute significantly to differentiate the cells in the group from the rest of the cells, yielding more succinct group definitions. Using the open source R software DepecheR on RNA sequencing data and mass cytometry data, the number of defining markers were reduced up to 1000-fold, thereby increasing interpretability vastly, while maintaining or improving the biological relevance of the groups formed compared to state-of-the-art algorithms.

Introduction

Since the introduction of the first single colour flow cytometers in the 1960s, there has been a remarkable increase in the complexity of data that can be generated with single-cell resolution. Currently, flow and mass cytometers able to simultaneously assess up to 40 cellular traits are becoming widely available [1]. In parallel, the development of high-throughput sequencing technology has facilitated deep single-cell transcriptomic analyses [2]. Furthermore, development of high-resolution single-cell proteomic analyses are underway [3].

These technological advances necessitate new computational approaches to analyses of multi- and megavariate single cell data [4–7]. Previous algorithms have contributed to automating analyses, thereby enhancing reproducibility and avoiding a need for *a priori* biological knowledge for design of manual gating analysis strategies. Automated analysis algorithms, not restricted to uni- or bivariate displays of the data, have also made it possible to display much more of the information embedded in multivariate data. To date, however, manual gating strategies are still dominantly used, which in part is likely due to that it is easy to interpret what population a certain gate refers to, as it is defined by few markers. In an attempt to combine the objectiveness and reproducibility of automated analysis pipelines with the high interpretability of manual gating strategies, we have developed an algorithm termed Determination of Essential Phenotypic Elements of Clusters in High-dimensional Entities (DEPECHE). DEPECHE simultaneously clusters and simplifies the data by identifying the variables that contribute to separate individual clusters from the rest of the data. We have implemented DEPECHE in R (in the open source package DepecheR), providing a complete software suite for statistical analysis and visualization of single cell omics data.

Results and Discussion

DEPECHE uses a penalized k-means clustering algorithm, related to the standard k-means algorithm [8]. In penalized k-means, a penalty term is introduced to the clustering algorithm. The value of the penalty, λ, determines the clustering resolution. Low clustering resolution implies that few clusters defined by few variables (high sparsity) are produced, and vice versa [9] (see online methods). Note that if k is high enough not to be limiting, the resolution of the emerging clusters depends entirely on the magnitude of λ and not on k, since DEPECHE annihilates all clusters that are pulled to the origin by the penalty. In DEPECHE, the penalty λ is tuned to identify the most *reproducible* clustering resolution, here termed the "optimal resolution"[10]. To illustrate what we mean by reproducible, we constructed a show case, featuring a bi-variate dataset D (Fig 1a). Visually, the dataset D contains three clusters, where the centers of the two larger clusters are located close to either axis. For these two clusters, one variable is sufficient to define their position. Now, if multiple datasets were generated from the same data source as D, for example by repeated experiments, we assume that they would contain the same clusters. Hence, imposing the optimal penalty λ_i (that corresponds to the optimal resolution of D) on all these datasets should ideally always result in the same clusters (high reproducibility). Contrarily, when clustering the same datasets with a penalty λ that differ significantly from the optimal penalty, the stochastic differences between the datasets are likely to induce solutions that deviate in cluster number, number of defining variables, and cluster center positions. In practice, DEPECHE tests a range of penalty values ($\lambda_1 < \cdots < \lambda_n$), each on a collection of dataset pairs which are generated by sampling N_R data points from D (Fig 1b) with resampling. The optimal resolution is defined as the penalty λ_i, which yields the lowest average variability within each dataset pair, as

measured by the Adjusted Rand Index (ARI) [11] . In our example, this corresponds to the penalty λ_i that yields 3 clusters, since 3 similar clusters are identified in each resampled dataset of D (Fig 1c). The penalties λ_1 and λ_n are considered suboptimal, since with these penalties, the stochastic differences in the resampled datasets lead to less coherent clustering results compared to results obtained with the optimal penalty λ_i. From here (Fig 1c) DEPECHE uses two alternative routes. If the number of data points in the dataset D is high (as default $> 10^4$), the most generalizable cluster centers that were produced using the optimal penalty are chosen (see online methods) and the data points of D are allocated directly to their closest cluster center (Fig 1d). If the dataset D has few data points, the full dataset D is clustered using the optimal penalty λ_i (Fig 1e).

Fig 1: Illustration of the DEPECHE workflow: a) The original dataset D. b) n resampled datasets with N_R data points per dataset, are generated by sampling data points from D with resampling. Each resampled dataset has a corresponding penalty λ_i ($i = 1,,,n$). c) Each dataset in b is clustered with sparse k-means, using its corresponding penalty λ_i. The red frame highlights the clustering with the strongest attractors, *i.e.* the most generalizable solution (see online methods). d, e) Finally, the full dataset is clustered by allocating each data point to its closest cluster center, using the most generalizable cluster center solution produced in b.

To evaluate how biologically accurate DEPECHE clustering is on mass cytometry data, a 32-variate mass cytometry bone marrow dataset [12] was clustered, and the overlap to 14 manually pre-defined cell populations was quantified using the ARI. With this dataset, DEPECHE identified 7 clusters at the optimal resolution,

corresponding to all large pre-defined cell populations and to agglomerates of smaller cell populations, rendering an average ARI of 0.96, where an ARI of 1 corresponds to exact reproduction and an ARI of 0 means that the produced clusters are no more accurate than random allocation. (Fig 2a-b, S Fig 1a). Furthermore, using DEPECHE, the number of variables defining each cluster was reduced from 32 to a range from 8 to 28, thereby enhancing interpretability (Fig 2d). When comparing to other state-of-the-art clustering algorithms [12–17], DEPECHE obtained similar ARI as the best algorithms for both the 32-variate dataset and another 14-variate, 24 population, mass cytometry dataset [18] (S Fig 2a, Table 1).

Table 1: background information on all datasets

Dataset	Data origin	n cells	n variables in analysis	n clusters in original
Levine	Mass cytometry	104184	32	14
Bendall	Mass cytometry	81747	14	24
Björklund	scRNAseq	648	35177	4
Biase	scRNAseq	56	19571	3
Deng	scRNAseq	268	13867	10
Goolam	scRNAseq	124	15487	5
Kolodziejczyk	scRNAseq	704	15117	3
Pollen	scRNAseq	301	13860	11
Yan	scRNAseq	90	13608	7

Single-cell transcriptomic datasets feature tens of thousands of variables. Thus, the need to exclude irrelevant variables is even more pressing, as compared to cytometry datasets. We therefore evaluated DEPECHE's ability to cluster and extract the key transcripts defining clusters of a previously published single-cell transcriptomic dataset (Fig 2d-f) [19]. In this dataset, a total of 648 ILC1, ILC2, ILC3 and NK cells from three donors' tonsils were index-sorted prior to RNA sequencing. Hence, these cell types, manually defined by protein expression, can be compared to clusters unbiasedly determined by RNA expression profiles [19]. In the DEPECHE analysis, no pre-selection of transcripts was performed, and hence, 35177 unique

160 transcripts were included for each of the 648 cells. With the optimal penalty λ, four
161 clusters were identified (Fig 2e). These corresponded well to the cell types as defined
162 by protein expression; 84, 97, 91 and 97 percent of ILC1, ILC2, ILC3 and NK cells
163 sorted into separate clusters, respectively (Fig 2d, e, S Fig 1b), leading to an average
164 ARI of 0.78. Notably, cluster 1-4, corresponding to ILC1, ILC3, NK cells and ILC2,
165 were defined by 27, 27, 108 and 10 transcripts, respectively (Fig 2f and Table 2),
166 leading to a 99.9% average decrease in the number of variables. The transcripts
167 identified to define the clusters in our analysis were among those most differentially
168 expressed according to the original study [19] (Fig 2f). Thus, by identifying a finite
169 number of variables, DEPECHE analysis increases interpretability and aides down-
170 stream analyses. When DEPECHE clustering was compared to that of state-of-the-art
171 algorithms [5–7] on the aforementioned dataset and six others (see Table 1), it
172 performed consistently well as indicated by ARI (S Fig 2b). Thus, when applied to
173 megavariate data, DEPECHE produces biologically relevant clusters and reduces the
174 complexity of the result thousand-fold.
175
176 **Fig 2: DEPECHE performance with real datasets with 32 or 35177 variables.** a-
177 b) bi-variate t-distributed stochastic neighbor embedding (tSNE) representation of the
178 32-variate mass cytometry data. A: distribution of manually defined cell populations
179 over the tSNE field. B: distribution of DEPECHE clusters over the tSNE field. c)
180 Heatmap showing which variables that define each cluster. Red color indicates a
181 higher expression in the cluster than the most common expression for all
182 observations. Blue color conversely indicates lower expression than the geometric
183 mean for all observations. Grey color indicates that the variable in question does not
184 contribute to defining the cluster. For Fig a-c all, 104184 cells have been clustered. d-

e) tSNE representation of the 137-variate data subset that could efficiently distinguish the clusters in the 35177-variate single-cell transcriptome dataset. d) distribution of the cell types defined by index-sorting and manual gating on protein expression profiles shown over the tSNE field. e) distribution of DEPECHE clusters over the tSNE field. f) Violin plots illustrating the overlap between the original analysis by Björklund *et al* and the DEPECHE analysis. For each subplot, the left and right side illustrate the distribution of the transcripts defining the clusters, and all other transcripts, respectively. The y-axis shows the log10 of the p-values in the original analysis adjusted for multiple comparisons. For Fig d-f, all 648 cells have been clustered.

Table 2: transcripts defining clusters in Björklund *et al* dataset

Cluster 1/NK cells		
Transcript	Log10 of adjusted original p-value	Cluster center
CMC1	-8.95	0.23
CST7	-8.95	0.17
GNLY	-8.95	1.82
GZMA	-8.95	0.23
GZMK	-8.95	0.09
KLRC1	-8.95	0.57
KLRD1	-8.95	1.89
KLRF1	-8.95	1.29
NKG7	-8.95	1.93
PRF1	-8.95	0.14

Cluster 2/ILC1		
Transcript	Log10 of adjusted original p-value	Clluster center
AE000661.37	-8.67	-0.08
CCR7	-9.25	0.69
CD27	-9.25	0.97
CD3D	-9.25	3.50
CD3E	-4.13	0.48
CD3G	-9.25	2.56
CD4	-9.25	0.55
CD6	-9.25	0.76
CNN2	-7.04	0.01
COTL1	-9.25	0.63
CTSW	-7.82	-0.02
FCER1G	-9.25	-0.81
KLRB1	-9.25	-0.96
LITAF	-9.25	0.08
LST1	-9.25	-0.57
RNU2-6P	-7.67	-0.07
RP11-466H18.1	0.00	0.20
SH2D1B	-9.25	-0.29
SIT1	-9.25	1.06
TC2N	-9.08	1.02
TNFRSF18	-9.25	-0.21
TOB1	-2.23	0.23
TRDC	-9.25	-1.71
TRDJ2	-9.25	-0.55
TYROBP	-9.25	-0.71
U2..37	-9.03	-0.30
U2..55	-9.16	-0.39

Cluster 4 (ILC2)		
Transcript	Log10 of adjusted original p-value	Cluster center
A2M	-7.08	0.23
AC092580.4	-9.17	-0.89
CD2	-9.17	-2.69
CD300LF	-9.17	-0.06
CD3E	-4.21	-0.40
EMP3	-2.86	0.31
FCER1G	-7.08	-0.59
GATA3	-8.33	0.34
GSN	-9.17	-0.40
HPGDS	-9.17	0.37
IL10RA	-8.84	0.03
IL17RB	-9.17	0.14
IL23R	-9.17	-0.47
IL2RB	-9.17	-0.64
IL32	-9.10	1.04
KLRC1	-9.17	-0.51
KLRG1	-9.17	0.50
KRT1	-9.17	0.21
SH2D1B	-9.17	-0.64
TESPA1	-6.02	0.01
TMIGD2	-9.03	-0.11
TRAC	-9.17	-0.54
TXK	-8.53	-1.21
TYROBP	-9.17	-1.40
VWA5A	-9.17	-0.32
XCL1	-9.17	-0.62
XCL2	-9.17	-0.12

Cluster 3/ILC3		
Transcript	Log10 of adjusted original p-value	Cluster center
A2M	-9.52	-0.04
AC092580.4	-9.66	0.78
ADAM10	-6.38	0.15
ADAM28	-9.66	0.05
AFF3	-9.66	0.80
AHR	-8.27	0.24
AMICA1	-9.66	1.33
ARL4C	-4.37	-0.02
ATP8B4	-9.66	0.10
BST2	-8.28	0.44
C1orf162	-8.83	-0.69
CAT	-9.66	0.69
CD2	-7.77	0.44
CD300LF	-9.66	1.30
CD3D	-9.65	-0.36

Cluster 3/ILC3		
Transcript	Log10 of adjusted original p-value	Cluster center
CD3G	-9.66	-0.06
CD63	-6.03	0.21
CNN2	-9.66	-0.61
COTL1	-8.04	-0.25
CPNE7	-6.13	0.25
CTSA	-5.65	0.25
DCAF11	-6.48	0.04
DHRS3	-3.72	0.02
DOCK5	-9.66	0.30
ELOVL6	-9.66	0.31
EMP3	-7.26	-0.94
ENPP1	-9.66	0.17
FAIM3	-3.79	-0.23
FAM65B	-3.38	-0.47
FCER1G	-9.66	1.02
FES	-9.66	0.11
GIMAP4	-7.49	-0.38
GIMAP7	-5.46	-0.39
GSN	-9.66	1.24
HCST	-0.34	-0.11
HDAC9	-9.66	0.30
HLA-DRB1	-3.19	0.34
IL10RA	-9.66	-0.97
IL1R1	-9.66	0.42
IL23R	-9.66	1.67
IL32	-9.66	-0.85
IL4I1	-9.66	1.50
ISG20	-2.10	-0.01
ITGB2	-6.95	-0.76
ITM2C	-9.66	0.41
KIAA1324	-9.66	1.07
KIT	-9.66	1.30
KLRG1	-9.66	-0.26
KRT81	-9.66	1.00
LAT2	-6.18	0.16
LDHB	-9.35	-0.18
LINC00299	-9.66	1.43
LITAF	-9.66	-0.20
LST1	-9.66	1.68
LTA4H	-9.66	0.89
LY6E	-3.30	-0.27
MPG	-8.65	0.49
NCR2	-9.66	1.00
NKG7	-1.82	-0.04
NRP1	-9.66	0.35
NSMCE1	-9.66	0.64
OTUD5	-9.66	0.73

Cluster 3/ILC3		
Transcript	Log10 of adjusted original p-value	Cluster center
PCDH9	-9.66	1.25
PDCD4	-3.37	-0.02
PECAM1	-9.66	0.06
PRR5	-9.66	0.38
PTPN22	-4.79	0.05
RBPJ	-5.14	0.07
RHOC	-9.66	0.76
RP11-264B17.3..1	-1.41	-0.18
RP11-330A16.1	-9.47	0.01
RP11-466H18.1	-0.35	-0.02
RP11-845M18.6	-9.66	0.44
RPS8	-1.54	-0.06
S100A6	-6.94	-0.16
S1PR1	-9.66	-0.08
SELL	-8.49	-0.97
SELPLG	-7.57	-0.37
SERINC5	-9.66	-0.13
SH2D1B	-9.66	0.90
SLA	-5.90	0.16
SLC38A1	-1.48	-0.06
SLC4A10	-9.66	0.32
SORL1	-8.48	-0.23
SPINK2	-9.66	0.67
SPRY1	-8.67	0.11
STARD3NL	-9.66	0.23
TC2N	-6.72	-0.66
TCIRG1	-7.11	0.10
TLE3	-9.66	0.06
TMIGD2	-9.66	1.09
TNFRSF18	-9.66	0.79
TNFRSF25	-3.37	0.30
TNFRSF4	-7.13	0.04
TNFSF11	-9.66	0.31
TNFSF13B	-9.66	1.02
TOX	-9.66	0.35
TOX2	-9.66	0.69
TRAC	-0.80	0.46
TRAJ45	-8.56	0.70
TRAT1	-7.81	-0.12
TRDJ2	-6.30	0.07
TRGJP1	-7.31	0.11
TXK	-5.59	0.14
TYROBP	-9.66	1.15
VWA5A	-9.66	1.61
XCL1	-9.66	0.80
XCL2	-9.66	0.03

In conclusion, DEPECHE turns the penalized k-means methodology into a parameter free analysis technique guided by efficient calculation of the optimal clustering resolution. By doing so, it addresses the simultaneous problem of clustering and identification of biologically important variables that separate clusters. This is crucial in order to comprehend the noisy and often over-complicated data generated with current single cell technologies.

Methods

Clustering with DEPECHE

Clustering in DEPECHE is performed using a penalized version of the k-means algorithm, which is related to the k-means algorithm[8]. In this section, the k-means algorithm is outlined first, followed by an explanation of how it is extended to penalized k-means.

The k-means algorithm clusters data by fitting a mixture of normal distributions to the data with k equal mixture components and unit variance. Formally, k d-dimensional cluster centers, denoted $\mu_{i,j}$ where $i = 1 \dots k$ and $j = 1 \dots d$, are fitted to the n d-dimensional datapoints $x_{l,j}$, where $l = 1 \dots n$, by maximizing the score function

$$Q = \sum_{i=1}^{k} \sum_{l=1}^{n} z_{i,l} \sum_{j=1}^{d} \left(x_{l,j} - \mu_{l,j}\right)^2, \qquad (1)$$

where $z_{i,l}$ is 1 if the lth data point belongs to the ith cluster and zero otherwise. The score Q is optimized using an Expectation Maximization (EM) algorithm [20], *i.e.* so called E- and M-steps are iterated alternatingly until the score Q stops improving. In the E-step, the allocation variables $z_{i,l}$ are updated so that each data point is allocated to its closest cluster. In the M-step, each cluster center $\mu_{i,j}$ is moved to the center of the data points allocated to it. When no more reallocation occurs in the E-step, the algorithm has converged.

In order to reduce the influence of uninformative dimensions that only contribute with noise, penalized k-means introduces an L1-penalty for each element

of each cluster center $\mu_{i,j}$ to the optimization objective. Formally, the score function Q in Eq. (1), is updated:

$$Q = \sum_{i=1}^{k}\sum_{l=1}^{n} z_{i,l}\sum_{j=1}^{d}\left(x_{l,j} - \mu_{l,j}\right)^2 - \lambda\sum_{i=1}^{k}\sum_{j=1}^{d}|\mu_{i,j}|\,, \qquad (2)$$

where λ is a positive penalty term. The additional term in the score function, introduced in Eq. (2) results in a change in the M-step of the original EM-algorithm of the k-means algorithm. Keeping $z_{i,l}$ for all l fixed and optimizing Q with respect to $\mu_{i,j}$, the M-step is:

$$\mu_{i,j} = sign\left(\frac{\sum_{l=1}^{n} z_{i,l}x_{l,j}}{\sum_{l=1}^{n} z_{i,l}}\right)\cdot \max\left(\left|\frac{\sum_{l=1}^{n} z_{i,l}x_{l,j}}{\sum_{l=1}^{n} z_{i,l}}\right| - \frac{\lambda}{2\sum_{l=1}^{n} z_{i,l}}, 0\right)\,. \qquad (3)$$

Depending on the choice of the penalty parameter λ, some components of some clusters centers will be set to 0 in the M-step. Note that penalized k-means with penalty $\lambda = 0$ reduces to the original k-means algorithm.

In DEPECHE, cluster centers that are moved to the origin in the M-step are eliminated and not assigned any data points in the E-step. Due to the elimination of clusters, the number of produced clusters is independent of k and dependent on the penalty λ as long as at least one cluster is eliminated. In DEPECHE, k is always chosen to be so large that at least one cluster is eliminated.

Eq. (2) is a special case of the penalized model based clustering algorithm by Pan and Shen with unit variance and equal mixture components [9]. By imposing the penalty for each dimension and each cluster, penalized k-means identifies the dimensions that do not distinguish a particular cluster from the rest of the data, thus leaving these dimensions out of the definition of that cluster. This differs from the sparse k-means algorithm by Witten and Tibshirani [21] and the regularized k-means

245 algorithm by Sun et al [10], that only identify dimensions that do not contribute to
246 distinguish any cluster from the rest of the data.

247 Penalized k-means, as well as k-means, relies on a procedure for initializing
248 the positions of the cluster centers. Cluster initialization is particularly delicate in
249 DEPECHE, due to the elimination of clusters at the origin in the E-step. Poor
250 initialization of the clusters might lead to elimination of too many clusters in the early
251 E-steps, yielding fewer clusters in the end result than necessary to optimize Q. To
252 avoid early elimination of clusters, DEPECHE initializes the cluster positions using
253 the seed generation algorithm of k-means++ by Arthur and Vassilvitskii[22] and
254 always starts clustering with penalty $\lambda = 0$. The penalty is then increased linearly
255 over a number of E-steps until it reaches the predetermined value.

256 The EM-algorithm guarantees convergence to an optimum of the score Q, but
257 not necessarily to the global optimum. In order to diminish the influence of the
258 starting state, the EM-algorithm is run several times with random initialization, and
259 the solution with optimal Q is chosen. In addition, k is set considerably higher than
260 the expected number of final clusters, which also diminishes the sensitivity to the
261 starting state. In the extreme case where k is set equal to the number of data points n,
262 the outcome of penalized k-means is deterministic.

263

264 **Tuning the penalty**

265 In this section, we describe the optimization scheme which is used for tuning the
266 linear penalty λ. The outline of the algorithm:

267

268 1. Choose a range of penalty terms $\lambda_i, i = 1..N_\lambda$ that are considered for
269 clustering the dataset D

2. Create 2 datasets per penalty term λ_i, called $D_{1,i}$ and $D_{2,i}$, by sampling N_r data points from D with replacement.
3. Run the penalized k-means algorithm on the datasets $D_{1,i}$ and $D_{2,i}$, yielding sets of cluster centers, denoted $\mathrm{M}_{1,i}$ and $\mathrm{M}_{2,i}$.
4. Create the partitions $P_{1,i}$ and $P_{2,i}$, by allocating all data points of the dataset D to their nearest cluster center of the sets $\mathrm{M}_{1,i}$ and $\mathrm{M}_{2,i}$.
5. Determine the Adjusted Rand Index (ARI), denoted $r(\lambda_i)$ from $P_{1,i}$ to $P_{2,i}$ [11].
6. Repeat step 2-5 times and average the obtained ARIs $r(\lambda_i)$ penalty wise until a stopping criteria regarding the statistical certainty of the obtained ARIs $r(\lambda_i)$ is met.
7. Choose the optimal penalty λ_i, which is the penalty with the largest ARI $r(\lambda_i)$.

Some remarks to the parameter tuning procedure: The repetition Step 6 is necessary, since the obtained ARI $r(\lambda_i)$ is a random variable, due to the random procedure for creating the datasets $D_{1,i}$ and $D_{2,i}$ and the random procedure for initializing the penalized k-means algorithm. DEPECHE uses two stopping criteria: The first criterion creates an interval of width 2 standard errors around the obtained mean of $r(\lambda_i)$ and checks if the interval around the optimal ARI $r(\lambda_i)$ has a zero overlap with the other intervals. The second criterion checks whether the standard error of the mean of $r(\lambda_i)$ for the optimal penalty λ_i is below a threshold.

Step 2 requires a samples size N_r. A natural choice is to set N_r equal to the number of data points, *n*. However, in cases where *n* is very large, so that the computational load of the optimization scheme becomes limiting, it is preferable to choose a smaller N_r.

In DepecheR, $N_r = 10^4$ by default, in case $n \geq 10^4$. Notice that when an optimal penalty λ_i is discovered using sample size $N_r \neq n$, the corresponding optimal penalty when sampling the full dataset D with magnitude n is (approximately) $\lambda_i \cdot \frac{n}{N_r}$, since the attraction force of a cluster is proportional to the number of data points in it.

Exact calculation of the ARI in step 5 is computationally intractable for large datasets. Therefore, DEPECHE relies on an approximate ARI computation, based on 10^4 random pairs of data points.

Simultaneous Clustering and Parameter Tuning

For very large datasets ($n > 10^8$), not only the penalty optimization, but also the final clustering once the optimal penalty has been found may be computationally intractable. However, increasing the size of the dataset, does not necessarily lead to an increase in number of clusters at the optimal resolution. In this case, it is feasible to cluster a subset of the full dataset D to obtain cluster centers M and then allocate the remaining data points of D to their closest clusters in M. This boosts computational efficiency since allocation imposes a much smaller computational load than clustering. Since several subsets of D are produced and clustered during the tuning of the penalty parameter λ, it seems natural to retrieve cluster centers M that were produced during the parameter tuning and use them to cluster D.

When picking a set of cluster centers M from the penalty tuning, the question arises which set of centers M to take, since several sets of centers, denoted $M_{i,l}$ ($l = 1,,,p$), are produced for the optimal penalty λ_i. In DEPECHE, the centers $M_{i,j}$ that have the strongest similarity (on average) to the remaining $p - 1$ centers is chosen and is referred to as the most generalizable cluster set. The level of similarity between the

centers $M_{i,j}$ and $M_{i,l}$ is quantified using the ARI between the partitions $P_{i,j}$ and $P_{i,l}$, induced by allocating each data point of D to its closest cluster center in $M_{i,j}$ and $M_{i,l}$ respectively.

Empiric Performance of the Penalty Tuning Scheme. Roughly speaking, DEPECHE combines a flavored penalized k-means algorithm with a parameter tuning scheme, which identifies an optimal resolution. A naturally arising question is then whether the parameter tuning scheme is able to determine a biologically relevant resolution or if other penalized k-means clustering resolutions outperform the resolution chosen by DEPECHE. Using a range of datasets (Table 3), the biological relevance (measured in ARI to the manually curated solution) of the optimized DEPECHE partitions were compared to the biologically optimal partition among all partitions generated with 20 repetitions on each of a range of 11 penalties per dataset. Overall, the DEPECHE resolution-selection showed close to optimal performance, as the selected solutions only had a median of 0.02 lower ARI to the gold standard (range 0-0.065) than the best possible solution with all penalties (Table 3).

Table 3: ARI between DEPECHE partitions and golden standard partitions

Dataset	Median ARI in supplementary figure 2	Maximal ARI with any penalty	Difference
Levine	0.961	0.975	0.015
Bendall	0.841	0.873	0.032
Biase	1	1	0
Björklund	0.782	0.842	0.06
Deng	0.827	0.848	0.021
Goolam	0.629	0.639	0.009
Kolodziejczyk	0.992	1	0.008
Pollen	0.863	0.928	0.065
Yan	0.626	0.691	0.064

Scaling and Centering the Data

The clusters produced by DEPECHE, as well as their interpretation, depends on the scaling and centering of the data. The scaling determines the relative importance of the measured variables, where variables with a larger spread have stronger influence on the clustering. The centering defines where zero occurs in each variable, thereby influencing the clustering results due to the linear penalty.

DEPECHE is applicable to a large range of datasets where the numbers of dimensions, d, and the number of data points, n, can vary with many orders of magnitude. The differing characteristics of these datasets require different treatments with respect to scaling and centering.

Scaling. Empirically, a majority of single-cell transcriptome datasets tend to have a few variables where the variance is many orders of magnitude greater than in the other variables. In this case, the high-variance variables will *de-facto* determine the clustering, implying that the clustering will fail to take the majority of the measured information into account. To even out the influence of these high-variance variables on the clustering outcome, the data is log transformed when such variables are present. In DepecheR, this data behavior is detected automatically by concatenating all variables into a one dimensional vector, for which the kurtosis is calculated. A high kurtosis, indicates that the variables differ greatly in their internal variance. For datasets with low kurtosis, refraining from the log transform is preferable, since transformation distorts the information.

Centering. Centering the origin to be close to the bulk of the data is preferable, in order to have all biological clusters at approximately the same distance from the

origin. Having some biological clusters close to the origin and some far off is often unwanted, since the linear penalty then imposes a preference for creating clusters close to the origin. Apart from influencing the clustering, the centering also determines the interpretation of the obtained sparsity. Just as for scaling, which centering scheme to apply depends on the dataset.

For low dimensional datasets (n>100), DEPECHE applies maximal density centering, which sets the zero in each dimension to coincide with the highest data density. The density it computed by collecting the data in equally spaced bins (default number of bins in DepecheR is the number of data points n divided by 50), where the bin with the highest number of data points has the highest density. Using this scheme, sparsity (*i.e.* that a variable is non-contributing to the definition of a cluster) is interpreted as that the data points in the cluster do not deviate from the most common outcome. It also ensures that the origin is relatively close to the bulk of the data, since it is located at the most common outcome for each variable respectively. The benefit of this scheme is that it boosts sparsity, by declaring the most common outcome non-defining. However, for high dimensional datasets ($n \geq 100$), maximal density centering can push the origin so far away from the center of mass of the dataset, that the penalty starts to impose an unwanted, artificial influence on the clustering, hampering the biological relevance of the clusters. To avoid this, DEPECHE imposes a mean centering scheme for such datasets, which locates the origin at the center of mass of the dataset.

A potential complication, related to centering, occurs when a biologically relevant cluster is located very close to the origin, since DEPECHE creates no clusters in the origin and will then force the cluster to merge with other clusters. However, this scenario was never detected in real data.

Experimental procedures

Preprocessing of mass cytometry data. The benchmark datasets from Levine *et al* [12] and Bendall *et al*[18] were transformed using the flowTrans package [23] before used in any clustering algorithm.

Preprocessing of single-cell transcriptomic data. The dataset from Björklund *et al* [19] was normalized using the sva package [24] as in the original manuscript. For this dataset, doublet variables were removed, lowering the number of variables from 64443 to 35177.

The gold-standard datasets used for benchmarking in the publication by Kiselev *et al* [5] were obtained in a pre-processed state. Before clustering with any algorithm, the gene filter function used in the sc3 package was used [5], with settings removing the genes that were expressed in more than 90% of the cells. This resulted in the number of transcripts presented in Table 1 (range 13608-19571 transcripts).

Code availability

All code necessary to generate the figures and tables in the manuscript are included in supporting code 1. The software package DepecheR is available for download at (https://github.com/theorell/depecher).

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Author contributions

A.T. drafted mathematical models, co-wrote the software implementation and the manuscript, Y.T.B. co-wrote the manuscript, J.T. co-wrote mathematical models and drafted software implementation and the manuscript.

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Supporting information

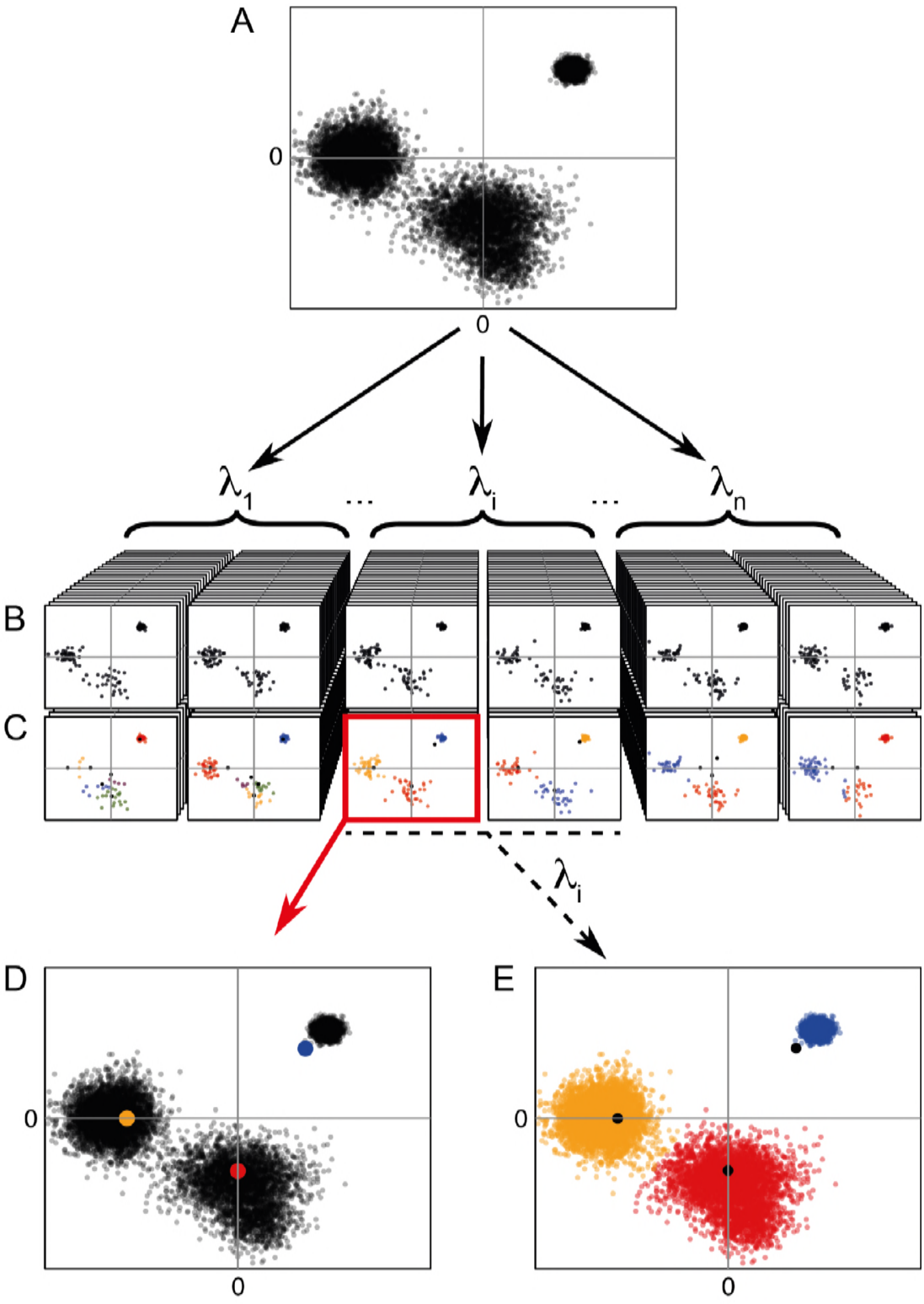
S1 Fig Heatmaps comparing the golden standard partitions to the DEPECHE partitions for a) the 32-variate Levine dataset and b) the 35166-variate Björklund dataset. Red color indicates large overlap, blue color indicates low overlap between a gold standard-vs-depeche cluster pair.

S2 Fig Algorithm comparisons. For all graphs, the x-axis shows the algorithms and the y-axis shows the Adjusted Rand Index comparing the clustering result with the golden standard clustering. a) Subsamples with 20000 unique cells from two mass cytometry datasets published by Levine *et al* and Bendall *et al* were clustered with DEPECHE and six previously published algorithms. For each dataset and algorithm, clustering was performed on 20 unique subsamples. For flowClust, flowPeaks and SamSPECTRAL, that do not perform internal parameter tuning, a range of parameter values were evaluated and the parameter value sets generating the highest ARI values were selected for display. b) The full Björklund dataset, as well as six other datasets previously used for benchmarking by Kiselev *et al* were clustered 20 times with

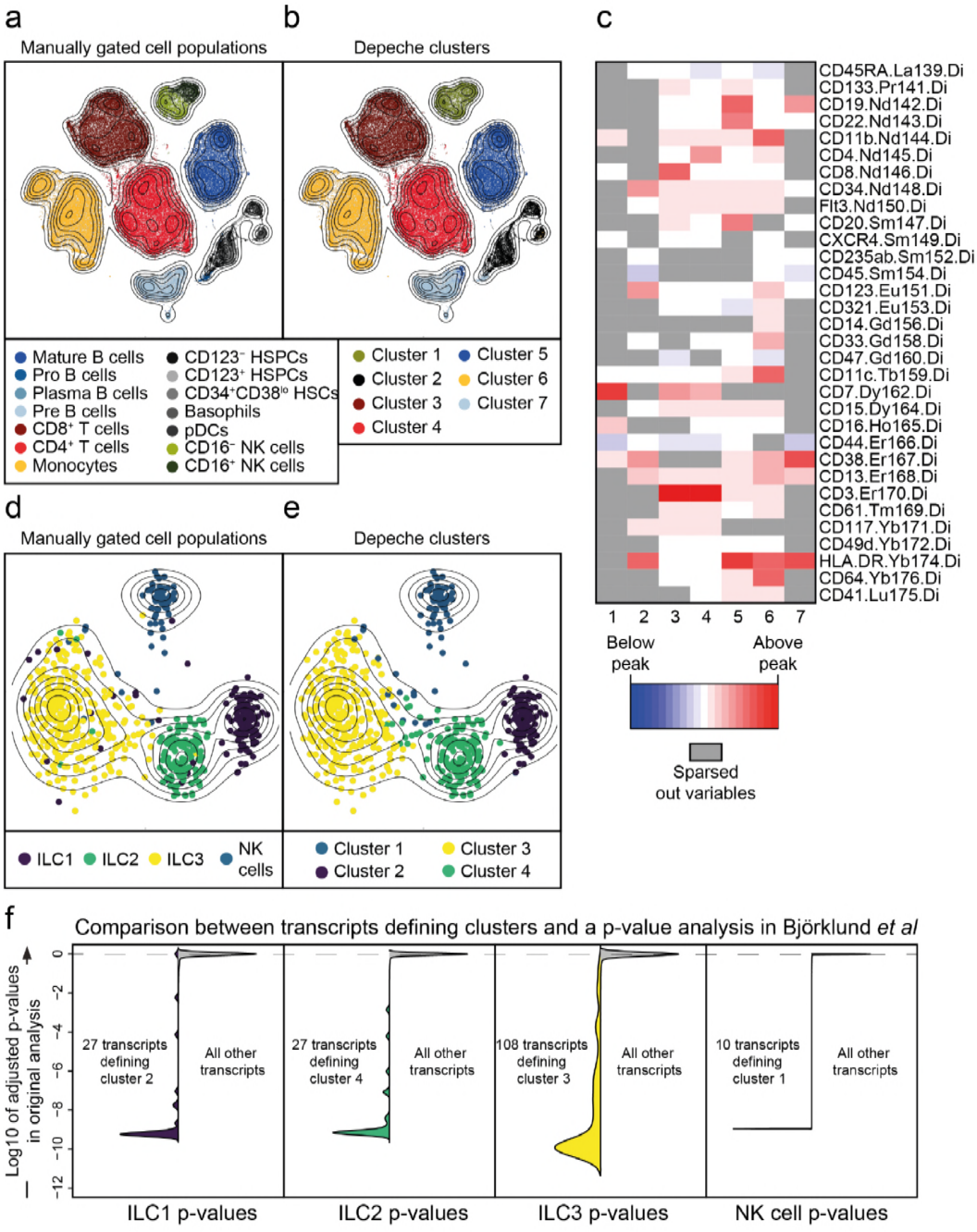
DEPECHE and three other algorithms. The Björklund dataset was normalized to reduce batch effects, with the procedure described in the original publication. These six datasets were also automatically log2-transformed within DEPECHE, and thus, log2-transformation was applied also for Sincera and pcaReduce, whereas sc3 was fed both log2- and untransformed data. The lower and upper hinges of all boxplots extend to the 25:th and 75:th percentile, whereas the line in the middle describes the median. The whiskers extend to the lowest and highest value no further than 1.5 times the distance between the 25:th and 75:th percentile. Outside of this range, the observations are considered outliers and are shown as dots.

S1 File. The DepecheR software, for the review phase.

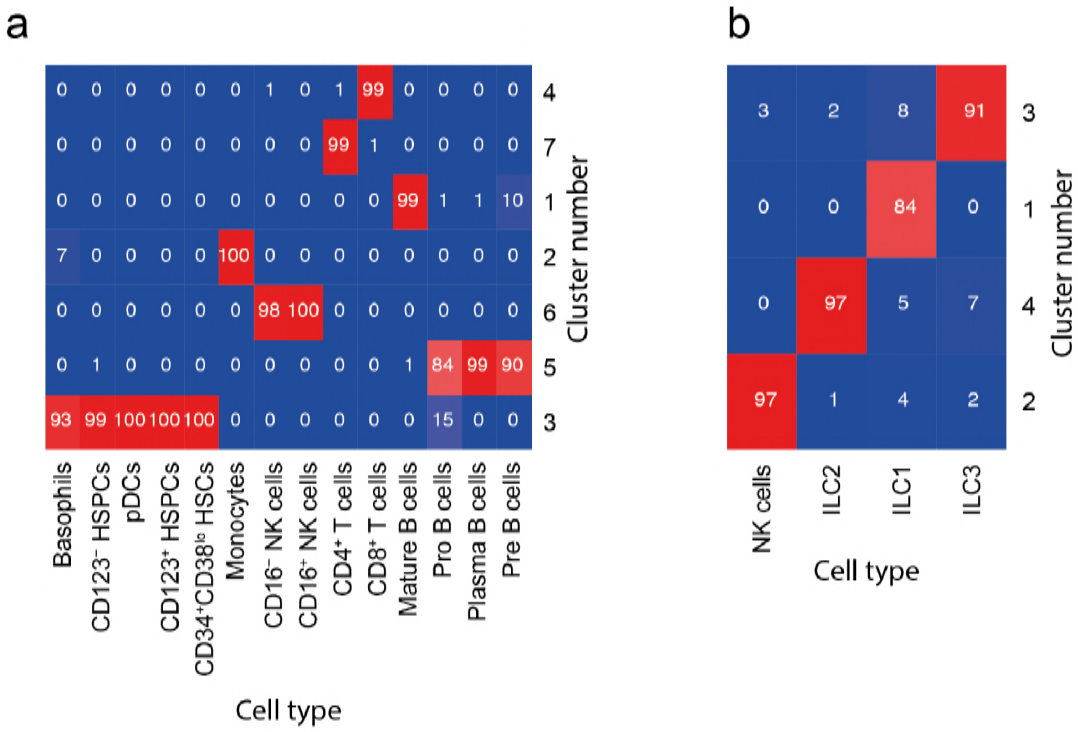
S2 File. The code needed to generate all figures, for the review phase.



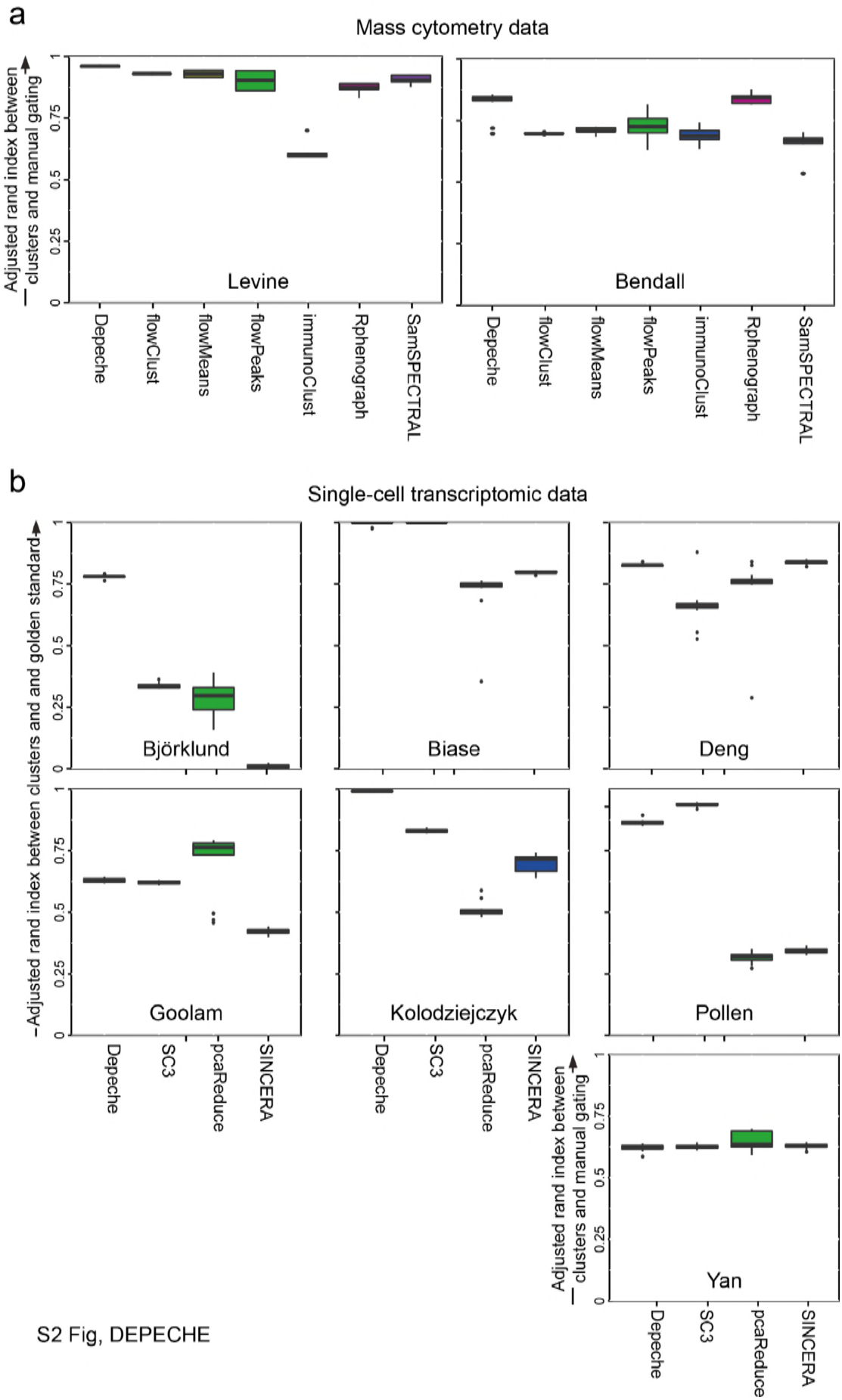
527 Figure 1, DEPECHE



528 Figure 2, DEPECHE



529 S1 Fig, DEPECHE



S2 Fig, DEPECHE