BEARscc: robustness of single-cell clusters determined using simulated technical replicates

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ABSTRACT

Technical variance is a major confounding factor in single-cell RNA sequencing, not least because measurements on the same cell are not replicable. We developed BEARscc, a tool that simulates experiment-specific technical replicates based on a probabilistic model of technical variance trained on RNA spike-in measurements. We demonstrate that the tool improves the unsupervised classification of cells and aids the interpretation of single-cell RNA-seq experiments.

Introduction

Single cell messenger RNA sequencing (scRNA-seq) can be used to establish an atlas of functional subtypes of cells in normal tissue[1] or to identify subpopulations of cells relevant to diseases such as cancer[2]. However, technical variability poses a major hurdle in the analysis of scRNA-seq data[3,4]. Read count measurements often vary considerably as a result of stochastic sampling effects, arising from the limited amount of starting material[3,4]. Frequently, expressed transcripts are not amplified during library preparation (the "drop-out" effect), resulting in a false-negative observation[3,4]. In addition to random technical variability, systematic variation due to differences in sample processing is a common problem. Batch-dependent differences in cDNA conversion, library preparation and sequencing depth can easily mask biological differences among cells. It was recently reported that the interpretation of many published scRNA-seq results might have been compromised by batch effects[5].

One approach to adjust for technical variation between samples that has become widely adopted is the addition of known quantities of synthetic RNA "spike-ins" to each cell sample before cDNA conversion and library preparation[6]. Several methods have been developed that make use of spike-ins to normalize read counts per cell before further analysis[7,8]. However, the use of spike-ins for normalizing read counts has been criticized for exacerbating the confounding influence of cell size and RNA content[7,9].

Here we present BEARscc, an algorithm that instead uses spike-in measurements to model the distribution of experimental technical variation across samples in order to simulate realistic technical replicates. This approach facilitates the interpretation of single-cell experiments, and also represents a novel use for spike-in controls that is not subject to the same problems as per-sample normalization. In summary, running BEARscc consists of three distinct steps (Figure 1). First, an experiment-specific model of technical variability ("noise") is estimated using observed spike-in read counts (Figure 1, step 1). This model consists of two parts. First, expression-dependent variance is approximated by fitting read counts of each spike-in across cells to a mixture model (see Methods). Drop-out effects are then treated separately. The '*drop-out injection distribution'* models the likelihood that a given transcript concentration will result in a drop-out, and is taken from the observed drop-out rate for spike-ins of a given concentration. The '*drop-out recovery*

distribution' models the likelihood that a transcript that had no observed counts in a cell was a false negative, and its density is estimated from the drop-out injection distribution using Bayes' theorem.

In the second step, BEARscc applies this model to produce simulated technical replicates (Figure 1, step 2). For every observed gene count below which drop-outs occurred amongst the spike-ins, BEARscc draws from the drop-out injection distribution to assess whether to convert the count to zero. For observations where the count is zero, the drop-out recovery distribution is used to estimate a new value, given the overall drop-out frequency for the gene. All non-zero counts after drop-out processing are substituted with a value generated by the model of expression variability, parameterized to the observed counts for each gene. This procedure can be repeated any number of times to generate a collection of simulated technical replicates. Re-analyzing these replicates in the same way as the original observation can then reveal the robustness of the results to the modelled technical variation.

In the third step, we focus on clustering analysis. Each simulated technical replicate is clustered using the same algorithm parameters as for the original observation. An association matrix is created, where each element indicates whether two cells share a cluster identity (1) or cluster apart from each other (0) in a particular replicate (Figure 1, step 3). The association matrices are combined to form the *noise consensus matrix*. Each element of this matrix represents the fraction of simulated technical replicates that, upon applying the clustering method of choice, resulted in two cells clustering together (the *association frequency*). Finally, three metrics are calculated from the consensus matrix: *stability* is the average frequency with which cells within a cluster associate with each other across simulated replicates, *promiscuity* measures the association frequency between cells within a cluster and those outside of it, and *score* is the difference between *stability* and *promiscuity,* reflecting the overall "robustness" of the cluster.

A common challenge in cell type classification is determining the optimal number of clusters, k, into which cells are placed. Heuristics, such as the silhouette index or the gap statistic[13,14], are commonly used but fail to account for expected variance in the similarity between cells. BEARscc's *score* statistic provides an alternative approach to address this issue. Performing hierarchical clustering on the noise consensus matrix allows BEARscc to split cells into any number of clusters between 1 and N (the total number of cells). The clustering with a maximum *score* (within a biologically reasonable range) then represents the optimal trade-off between within-cluster stability and between clusters variability (see Methods).

To assess the accuracy of BEARscc, we diluted one RNA-seq library derived from bulk human brain tissue to single cell RNA concentrations and sequenced 48 of these samples with appropriate spike-ins. The mean and variance of the simulated counts produced by BEARscc closely matched the experimentally determined values (Figure 1, step 1 - top; Supplementary Figure 1a,b). We also observed that for 95% of the genes expressed in the library, the simulated drop-out rate changed by less than 9% (Figure 1, step 1 – bottom, Supplementary Figure 1c). Together, these results suggest that technical variation simulated by BEARscc closely resembles that observed experimentally. The simulated expression of genes with fewer than 1 observed count deviated slightly from the experimentally determined values, however such small expression differences are unlikely to be reproducible as they fall outside the dynamic range of any single cell experiment.

In addition to the diluted brain RNA samples, we sequenced 45 "blank" samples, which only contained spike-ins and trace amounts of environmental contamination, producing sporadic read counts. We clustered the technical replicate data and the blank cells with three widely used clustering algorithms (RaceID2[10], BackSPIN[11], and SC3[12]), expecting perfect separation of brain and blank samples. To avoid artifacts due to differences in amplification-dependent library size, we applied an adjusted cpm normalization[5], otherwise standard parameters were used for all three programs. As an alternative to BEARscc, we also tested a simple sampling approach where we repeatedly sampled half of all expressed genes and re-clustered the cells based on this subset (see Methods). Without BEARscc or sub-sampling, all three clustering algorithms were prone to create false-positive clusters (Figure 2a, Supplementary Figure 2a-c, top). In contrast, BEARscc provided a clear improvement over the original clustering and the sampling approach (Figure 2a). Overall, BEARscc separated brain tissue and blank samples correctly and eliminated spurious clusters that corresponded to batch effects (color bars atop Supplementary Figure 2a,c). Only in the case of RaceID2 three outlier cells were incorrectly identified to be "robust" (color bars atop Supplementary Figure 2b); the libraries for these three samples contained fewer than 1,000 observed transcripts, indicating that BEARscc is limited by RaceID2's oversensitivity to library size differences.

To test BEARscc on real biological data, we applied it on murine brain data from Zeisel *et al.*[11]. Based on the *score* statistic, BEARscc reduced the 24 clusters produced by BackSPIN into 11 clusters which corresponded well with the manually curated cell types described in the original publication (Adjusted Rand Index 0.72/0.55 for BEARscc/BackSPIN; Figure 2a (right), Figure 2b).

In a second evaluation, we re-analyzed murine intestinal data obtained by Grün *et al.*[10], clustered using RaceID2, as described in the original publication. BEARscc indicated that 219 out of 291 cells were robustly classified. However, clusters R1 and R2 exhibited markedly lower *scores* than the other main clusters (Supplementary Figure 3a). BEARscc's consensus matrix reveals high variability in clustering patterns of R1/R2 cells. Grün *et al.* suggest that R1 and R2 reflect closely related, undifferentiated cell types ("transit-amplifying" and "stem-like", respectively). Expression patterns of genes characteristic of the two clusters were highly similar (Supplementary Figure 4a), compared to those observed between R1 and the next-largest cluster R5 (Supplementary Figure 4b). In the case of R1 and R2, expression fold-change was reduced in technical replicates, frequently falling below the significance threshold. BEARscc directly visualized the fact that many cells in R1 and R2 cannot be reliably classified, but rather appear to represent a point on a gradient of differentiation between two cellular states. More work will be needed to fully determine how the differentiation state of stem-like cells is reflected by their transcriptome.

In summary, our results demonstrate that BEARscc reduces over-clustering, is able to identify biological cell groups in an unsupervised way and provides additional insights for the interpretation of single-cell sequencing experiments.

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FIGURES

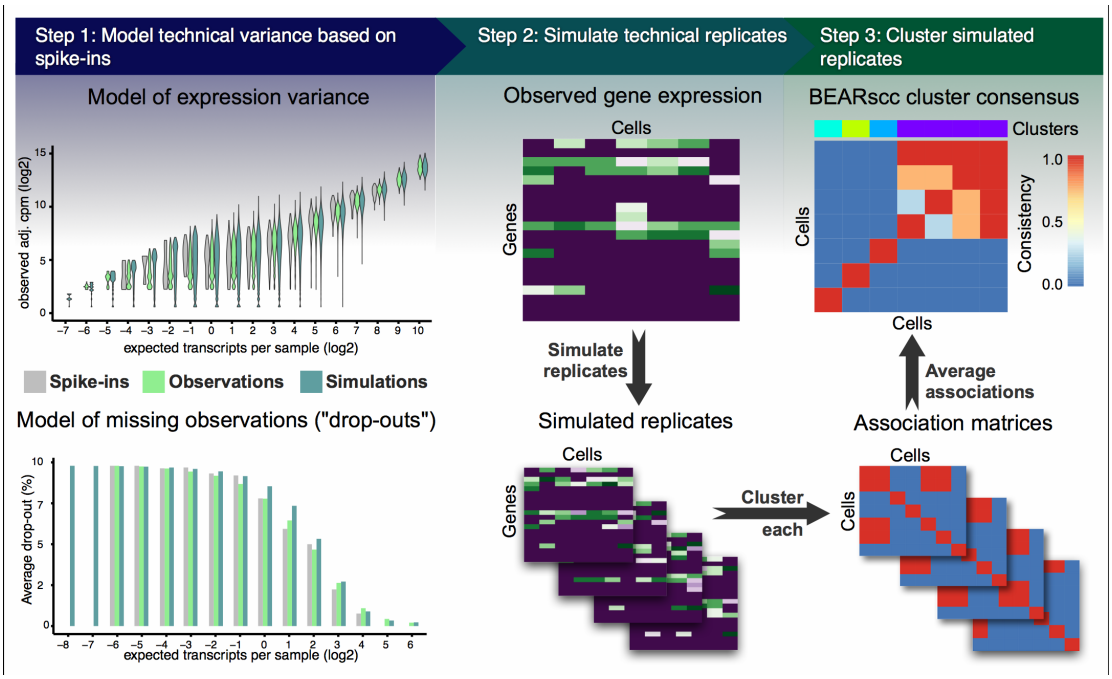

Figure 1 BEARscc algorithm overview. **Step 1,** Expected variance of gene expression between replicate experiments is estimated from the variation of spike-in measurements. Top: variation in spike-in read counts corresponds well with experimentally observed variability in biological transcripts (for details of control experiment see Methods) and read counts simulated by BEARscc. Bottom: Drop-out likelihood is modelled separately. **Step 2,** simulating replicates: an observed counts matrix (top) is transformed into multiple simulated technical replicates (bottom) by repeatedly applying the noise model derived in Step 1 to every cell in the matrix. **Step 3,** calculating a consensus: association matrices that result from clustering each simulated replicate (bottom) are averaged into a single *noise consensus matrix* that reflects the frequency with which cells are observed in the same cluster across all simulated replicates. Based on this matrix, a noise consensus cluster can then be derived (color bar above matrix).

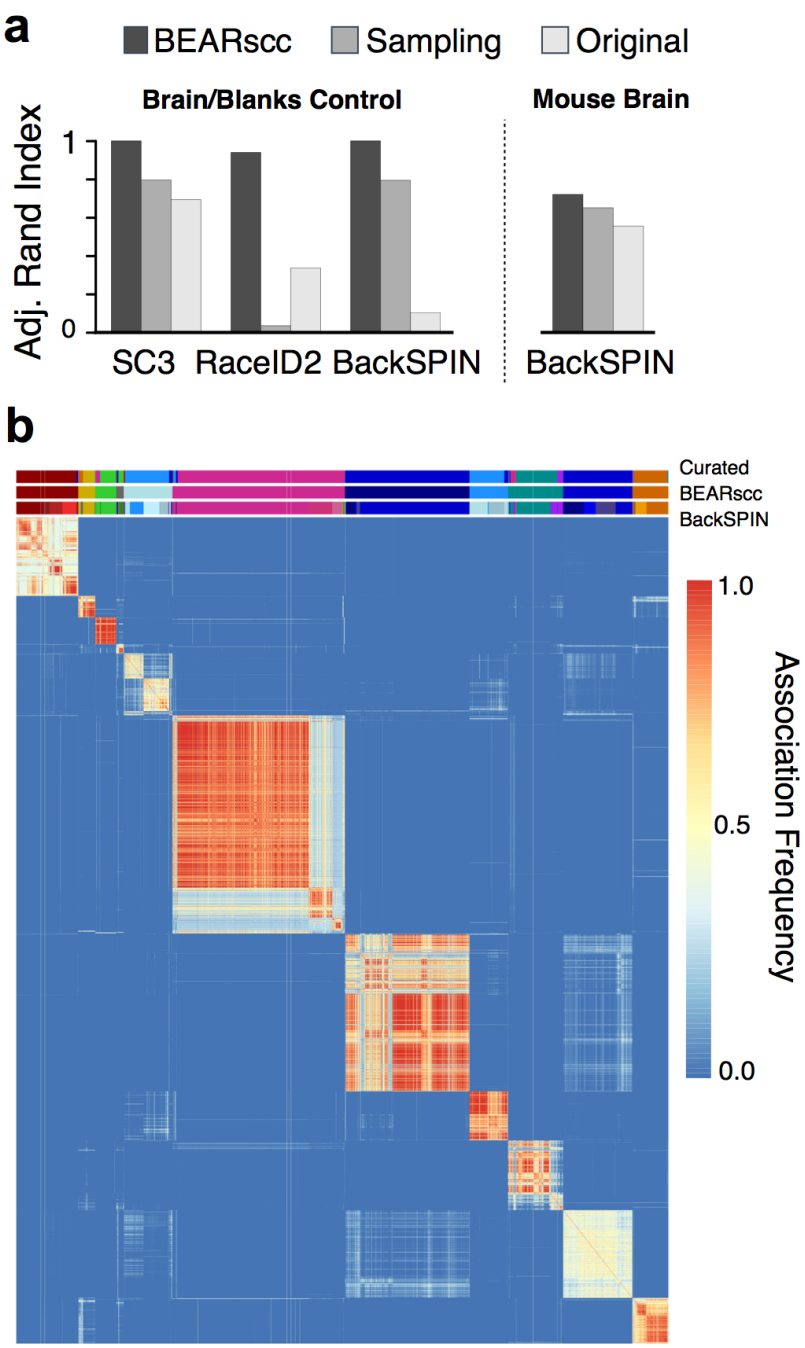

Figure 2 BEARscc improves clustering results and aids the interpretation of biological results. **a,** Comparison of clustering accuracy of control data (left) and murine brain data (right). Adjusted Rand index denotes agreement with the manually annotated grouping of samples (1: perfect, 0: no overlap). **b,** Example of a noise consensus matrix produced by BEARscc on data from murine brain cells clustered with BackSPIN. Above heatmap: manually curated clustering of cells (top), BEARscc consensus cluster (middle) and unsupervised BackSPIN clusters (bottom).

METHODS

Public data. Primary murine cortex and hippocampus single cell measurements for 3005 cells from Zeisel *et al.*[11] were retrieved from the publicly available Linnarsson laboratory data repository (http://linnarssonlab.org/cortex/). Primary murine intestinal single cell measurements of 260 cells from Grün *et al.*[1] were downloaded from the van Oudenaarden github repository (https://github.com/dgrun/RaceID).

Implementation. All scripts necessary for implementing BEARscc are available from our bitbucket repository as an installable R package (https://bitbucket.org/bsblabludwig/bearscc).

Algorithmic generation of simulated technical replicates. Simulated technical replicates were generated from the noise mixture-model and two drop-out models. For each gene, the count value of each sample is systematically transformed using the mixture-model, $Z(c)$, and the drop-out injection, $\Pr(X = 0 \mid Y = k)$, and recovery, $\Pr\left(Y_j = y \mid X_j = 0\right)$, distributions in order to generate simulated technical replicates as indicated by the following pseudocode:

```
FOR EACH gene, j
    FOR EACH count, c
        IF c = 0
            n ← SAMPLE one count, y, from Pr (Y_j = y | X_j = 0)
            IF n = 0
                c ← 0
            ELSE
                c ← SAMPLE one count from Z(n)
            ENDIF
        ELSE
            IF c ≤ k
                dropout ← TRUE with probability, Pr(X = 0 | Y = k)
                IF dropout = TRUE
                    c ← 0
                ELSE
                    c ← SAMPLE one count from Z(c)
                ENDIF
            ELSE
                c ← SAMPLE one count from Z(c)
            ENDIF
        ENDIF
        RETURN c
    DONE
DONE
```

Modelling noise from spike-ins. Technical variance was modelled by fitting a single parameter mixture model, $Z(c)$ to th spike-ins' observed count distributions. The

noise model was fit independently for each spike-in transcript and subsequently regressed onto spike-in mean expression to define a generalized noise model. This was accomplished in three steps:

1. Define a mixture model composed of *poisson* and *negative binomial* random variables: $Z \sim (1-\alpha) * Pois(\mu) + \alpha * NBin(\mu, \sigma)$

2. Empirically fit the parameter, α_i, in a spike-in specific mixture-model, Z_i, to the observed distribution of counts for each ERCC spike-in transcript, i, where μ_i and σ_i are the observed mean and variance of the given spike-in. The parameter, α_i, was chosen such that the error between the observed and mixture-model was minimized.

3. Generalize the mixture-model by regressing α_i parameters and the observed variance σ_i onto the observed spike-in mean expression, μ_i. Thus the mixture model describing the noise observed in ERCC transcripts was defined solely by μ, which was treated as the count transformation parameter, c, in the generation of simulated technical replicates .

In step 2, a mixture model distribution is defined for each spike-in, i: $Z_i(\alpha_i, \mu_i, \sigma_i) \sim (1-\alpha_i) * Pois(\mu_i) + \alpha_i * NBin(\mu_i, \sigma_i)$. The distribution, Z_i, is fit to the observed counts of the respective spike-in, where α_i is an empirically fitted parameter, such that the α_i minimizes the difference between the observed count distribution of the spike-in and the respective fitted model, Z_i. Specifically, for each spike-in transcript, μ_i and σ_i were taken to be the mean and standard deviation, respectively, of the observed counts for spike-in transcript, i. Then, α_i was computed by empirical parameter optimization; α_i was taken to be the $\alpha_{i,j}$ in the mixture-model, $Z_{i,j}(\alpha_{i,j}, \mu_i, \sigma_i) \sim (1-\alpha_{i,j}) * Pois(\mu_i) + \alpha_{i,j} * NBin(\mu_i, \sigma_i)$, found to have the least absolute total difference between the observed count density and the density of the fitted model, Z_i. In the case of ties, the minimum $\alpha_{i,j}$ was chosen.

In step 3, $\alpha(c)$ was then defined with a linear fit, $\alpha_i = a * log2(\mu_i) + b$. $\sigma(c)$ was similarly defined, $log2(\sigma_i) = a * log2(\mu_i) + b$. In this way, the observed distribution of counts in spike-in transcripts defined the single parameter mixture-model, $Z(c)$, used to transform counts during generation of simulated technical replicates:

$$Z(c) \sim (1-\alpha(c)) * Pois(c) + \alpha(c) * NBin(c, \sigma(c)).$$

During technical replicate simulation, the parameter c was set to the observed count value, a, and the transformed count in the simulated replicate was determined by sampling a single value from $Z(c = a)$.

Inference of transcript drop-out distributions using spike-ins. A model of the drop-outs was developed in order to inform the permutation of zeros during noise injection. The observed zeros in spike-in transcripts as a function of actual transcript concentration and Bayes' theorem were used to define two models: the '*drop-out injection distribution'* and the '*drop-out recovery distribution'*.

The drop-out injection distribution was described by $\Pr(X = 0 \mid Y = y)$, where X is the distribution of observed counts and Y is the distribution of actual transcript counts; the density was computed by regressing the fraction of zeros observed in each sample, D_i, for a given spike-in, i, onto the expected number spike-in molecules in the sample, y_i, e.g. $D = a * y + b$. Then, D describes the density of zero-observations conditioned on actual transcript number, y, or $\Pr(X = 0 \mid Y = y)$. Notably, each gene was treated with an identical density distribution for drop-out injection.

In contrast, the density of the drop-out recovery distribution, $\Pr(Y_j = y \mid X_j = 0)$, is specific to each gene, j, where X_j is the distribution of the observed counts and Y_j is the distribution of actual transcript counts for a given gene. The gene-specific drop-out recovery distribution was inferred from drop-out injection distribution using Bayes' theorem and a prior. This was accomplished in 3 steps:

1. For the purpose of applying Bayes' theorem, the gene-specific distribution, $\Pr(X_j = 0 \mid Y_j = y)$, was taken to be the the drop-out injection density for all genes, j.

2. The probability that a specific transcript count was present in the sample, $\Pr(Y_j = y)$, was a necessary, but empirically unknowable prior. Therefore, the prior was defined using the law of total probability, an assumption of uniformity, and the probability that a zero was observed in a given gene, $\Pr(X_j = 0)$. The probability, $\Pr(X_j = 0)$, was taken to be the fraction of observations that were zero for a given gene. This was done in order to better inform the density estimation of the gene-specific drop-out recovery distribution.

3. The drop-out recovery distribution density was then computed by applying Bayes' theorem:

$$(1)\ \Pr(Y_j = y \mid X_j = 0) = \frac{\Pr(X_j=0 \mid Y_j=y) * \Pr(Y_j=y)}{\Pr(X_j=0)}.$$

In the second step, the law of total probability, an assumption of uniformity, and the fraction of zero observations in a given gene were leveraged to define the prior, $\Pr(Y_j = y)$. First, a threshold of expected number of transcripts, k in Y, was chosen such that k was the maximum value for which the drop-out injection density was non-zero. Next, uniformity was assumed for all expected number of transcript values, y greater than zero and less than or equal to k; that is $\Pr(Y_j = y)$ was defined to be some constant probability, n. Furthermore, $\Pr(Y_j = y)$ was defined to be 0 for all $y > k$. In order to inform $\Pr(Y_j = y)$ empirically, $\Pr(Y_j = 0)$ and n were derived by imposing the law of total probability (2) and unity (3) yielding a system of equations:

$$(2)\ \Pr(X_j = 0) = \sum_{y=0}^{k} \{\Pr(X_j = 0 \mid Y_j = y) * \Pr(Y_j = y)\}$$
$$(3)\ \sum_{y=0}^{k} \{\Pr(Y_j = y)\} = \Pr(Y_j = 0) + k * n = 1$$

The probability that a zero is observed given there are no transcripts in the sample, $\Pr(X_j = 0 \mid Y_j = 0)$, was assumed to be 1. With the preceding assumption, solving for $\Pr(Y_j = 0)$ and n gives:

$$(4)\ n = \frac{1-\Pr(Y_j=0)}{k}$$

$$(5)\ \Pr(Y_j = 0) = \frac{\Pr(X_j=0)-\frac{1}{k}*\sum_{y=1}^{k}\{\Pr(X_j=0 \mid Y_j=y)\}}{1-\frac{1}{k}*\sum_{y=1}^{k}\{\Pr(X_j=0 \mid Y_j=y)\}}$$

In this way, $\Pr(Y_j = y)$ was defined by (4) for y in Y_j less than or equal to k and greater than zero, and defined by (5) for y in Y_j equal to zero. For y in Y_j greater than k, the prior $\Pr(Y_j = y)$ was defined to be equal to zero.

In the third step, the previously computed prior, $\Pr(Y_j = y)$, the fraction of zero observations in a given gene, $\Pr(X_j = 0)$, and the drop-out injection distribution, $\Pr(X_j = 0 \mid Y_j = y)$, were utilized to estimate with Bayes' theorem the density of the drop-out recovery distribution, $\Pr(Y_j = y \mid X_j = 0)$. During the generation of simulated technical replicates for zero observations and count observations less than or equal to k, values were sampled from the drop-out recovery and injection distributions as described in the pseudocode of the algorithm.

Observing real technical noise. Brain whole tissue total RNA (Agilent Technologies, cat 540005) was diluted to 10pg aliquots and added to 1µL. cDNA conversion, library preparation, and sequencing were performed by the Wellcome Trust Center for Human Genomics Sequencing Core. Blank samples were identically prepared with nuclease free water. Samples were pipetted into 96-well plates and treated as single cells using Smartseq2 cDNA conversion as described by Picelli *et al*[15] with minor modifications. The library was prepared using Fludigm's recommendations for Illumina NexteraXT at ¼ volume with minor modifcations, and sequenced on the Illumina HiSeq4000 platform. Raw reads were mapped to hg19 using STAR[16]. Exact position duplicates were removed, and features were counted using HTseq [17].

Clustering of counts data. BackSPIN, SC3 and RaceID2 were run according to algorithm-specific recommendations [10-12]. RaceID2 was allowed to identify cluster number under default parameters. For the brain and blanks control experiment data, RaceID2 was modified to skip normalization since scaled counts per million normalization had already been applied to the data set. The number of clusters, *k*, selected for SC3 clustering was determined empirically by selecting *k* with the optimal silhouette distribution across noise injected counts matrices.

Computation of consensus matrix. 100 simulated replicate matrices for *n* cells and *m* genes were clustered using the respective clustering algorithm (SC3, BackSPIN, RaceID2) as described above. Cluster labels were used to compute an *n* x *n* binary association matrix for each clustering. Each element of the association matrix represents a cell-cell interaction, where a value of 1 indicates that two cells share a cluster and a value of 0 indicates two cells do not share a cluster. An arithmetic mean was taken for each respective element across the resulting 100 association matrices to produce an *n* x *n* noise consensus matrix, where each element represents the fraction

of noise injected counts matrices that, upon clustering, resulted in two cells sharing a cluster.

Computation of BEARscc cluster metrics. To calculate cluster *stability*, the noise consensus matrix was subset to cells assigned to the cluster. The cluster *stability* was then calculated as the arithmetic mean of the upper triangle of the subset noise consensus matrix. To calculate cluster *promiscuity*, the rows of the noise consensus matrix were subset to cells assigned to the cluster and the columns are subset to the cells not assigned to the cluster. For clusters with as many or more cells assigned to them than not assigned, the *promiscuity* was defined as the arithmetic mean of the elements in the subset matrix. Otherwise, the columns were further subset to the same number of cells as were assigned to the cluster, where the cells outside of the cluster with the strongest mean association with cells inside the cluster are chosen. The *promiscuity* was defined as the arithmetic mean of the elements in this further subset matrix. Each cluster's *promiscuity* was subtracted from its *stability* to calculate cluster *score*.

Computation of BEARscc cell metrics. To calculate a cell's *stability*, the arithmetic mean was taken of that cell's association frequencies with other cell's within the cluster. To calculate a cell's *promiscuity*, there were two cases. For cells in clusters with as many or more cells assigned to them than not assigned, the *promiscuity* was the arithmetic mean of that cell's association frequencies with all cells not assigned to the relevant cluster. For cells in clusters of size n, with fewer cells assigned to them than not assigned, the cell's *promiscuity* was the arithmetic mean with the n cells not assigned to the cluster with the highest association frequencies. Each cell's *promiscuity* was subtracted from its *stability* to calculate cell *score*.

Estimation of cluster number *k*. In order to determine the cluster number, k, from the hierarchical clustering of the noise consensus, the resulting dendrogram was cut multiple times to form N clusterings with cluster numbers k=1 to k=N clusters. The average *score* metric was computed for each clustering, and k was chosen by taking the k with the maximum average *score* metric. Evaluating all possible k from 1 to the number of cells in the experiment is computationally expensive and unlikely to be biologically meaningful. In this work, N was capped at 0.1 times the number of cells in the experiment: N=10 for the brain and blanks control, N=30 for the murine intestine experiment, and N=300 for the murine brain data.

Gene sampling. For comparison with BEARscc, 100 subsampling iteration matrices for n cells and m genes were generated by sampling one half of expressed genes and clustered using the respective clustering algorithm (SC3, BackSPIN, RaceID2). For each dataset, genes were excluded with less than 25 total raw counts across all samples in the cohort. The remaining genes formed the sample space. In each subsampling iteration, one half of the genes were sampled without replacement, and their expression across cells was used as the counts matrix. Identically to the computation of the BEARscc noise consensus matrix, cluster labels were used to compute an n x n binary association matrix for each clustering, and an arithmetic mean was taken for each respective element across the resulting 100 association matrices to produce an n x n subsampling consensus matrix. Identically to BEARscc

analysis, the BEARscc *score* metric was used to determine cluster number *k*, and the resulting cluster labels for each dataset and algorithm were compared with BEARscc by computing the adjusted rand index for each with respect to the relevant ground truth.

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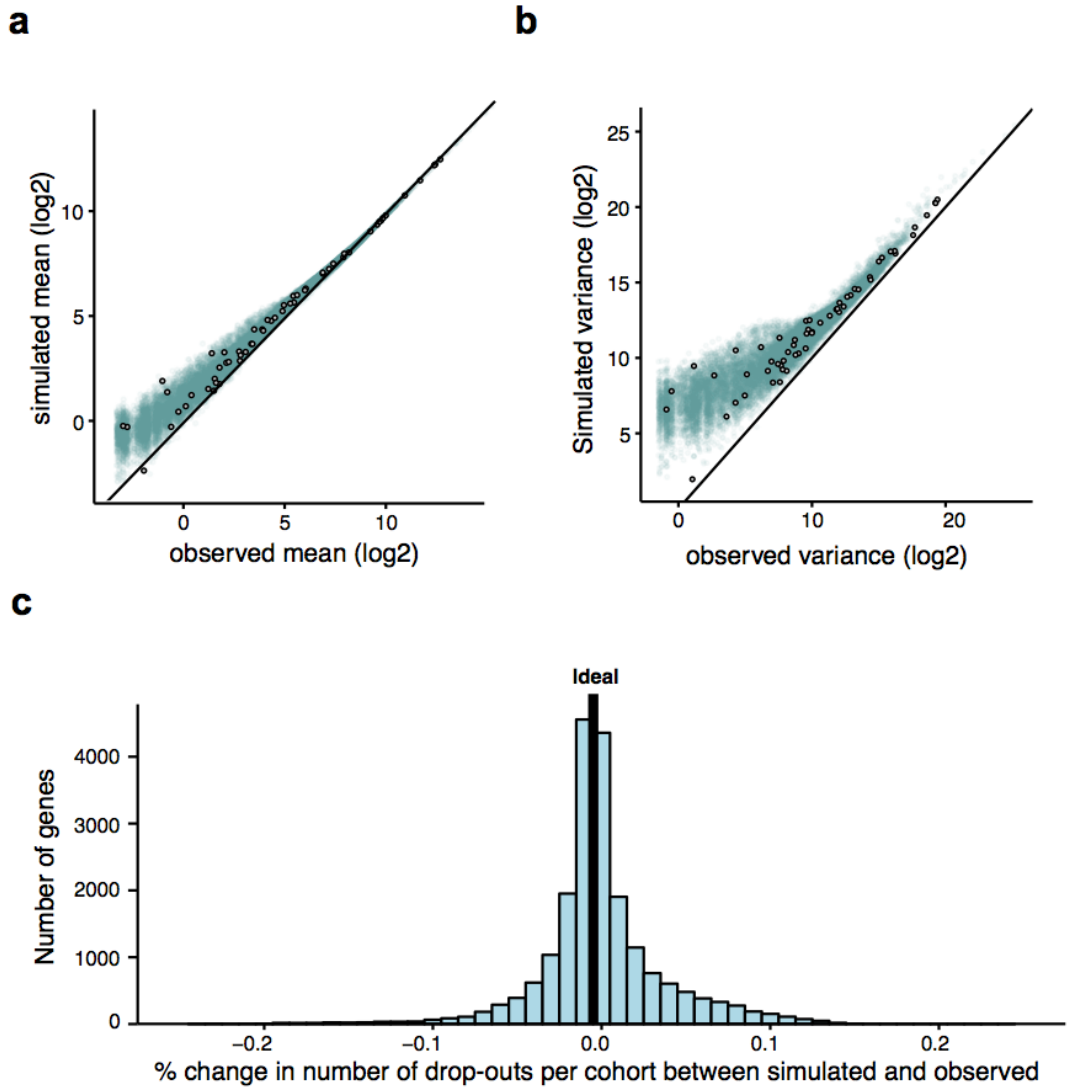
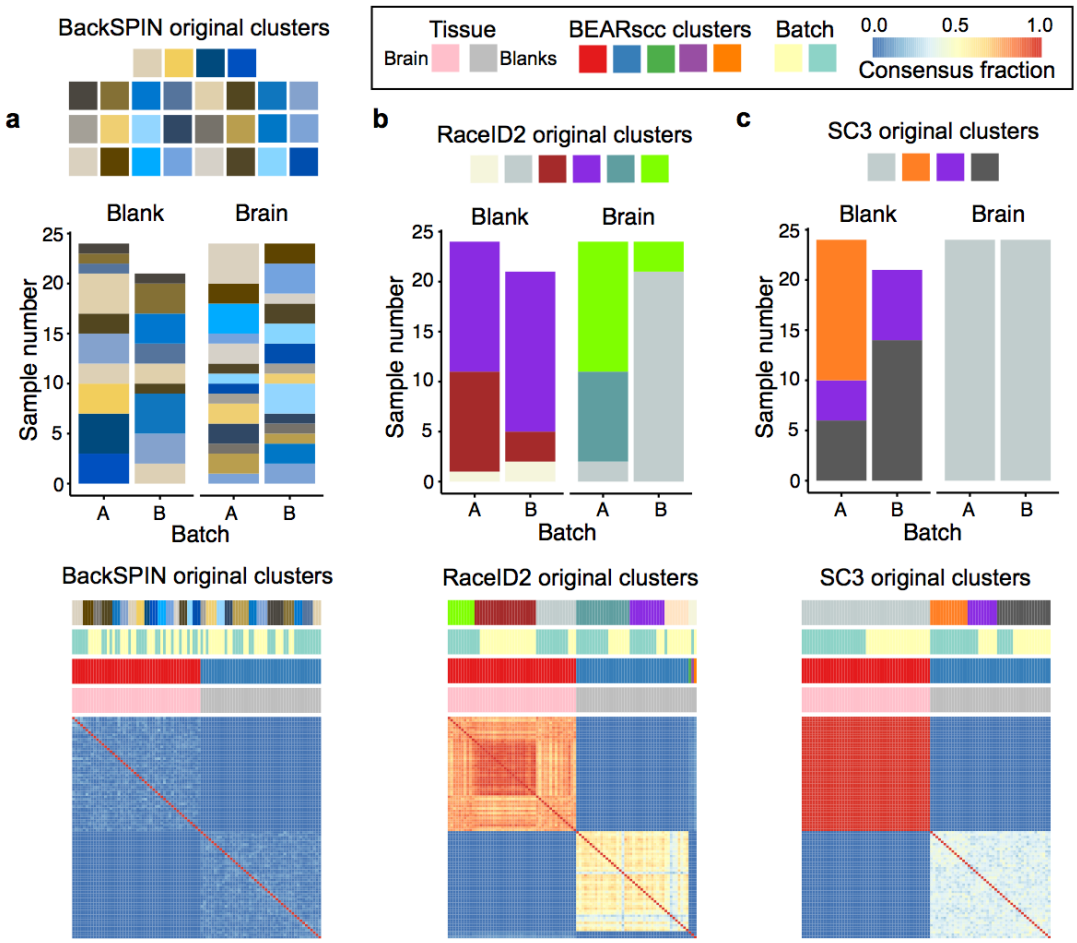
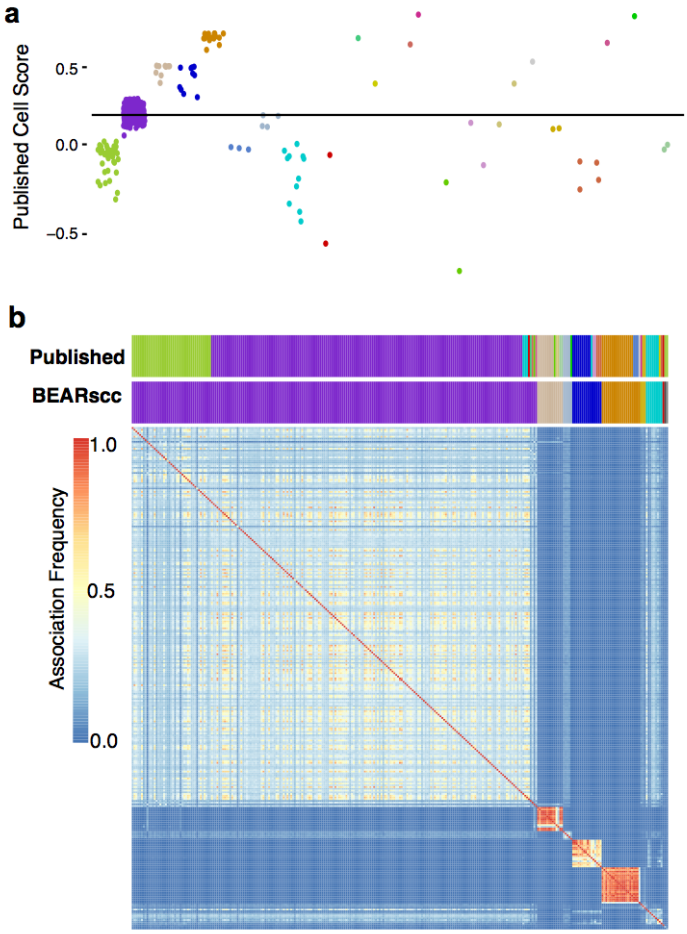
Author Contributions
D.T.S conceived and implemented the computational approach under the supervision of B.S.-B. M.W., R.O., and X.L. designed the initial experimental study that that led to the development of the presented approach. B.S.-B. and D.T.S. prepared the manuscript, with contributions for all authors.



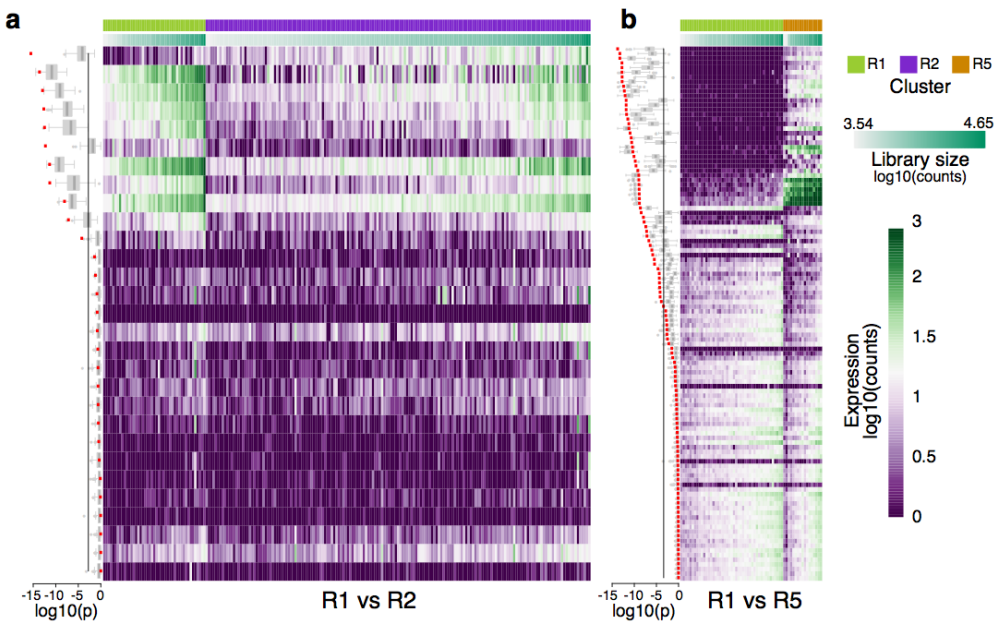
Supplementary Figure 1 BEARscc accurately models technical variability. Scatterplots of observed vs simulated expression **(a)** and variance **(b)**, based on data from brain RNA control experiment. ERCC spike-in values are circled in black. **c**, Difference between simulated and observed drop-out frequency across genes.



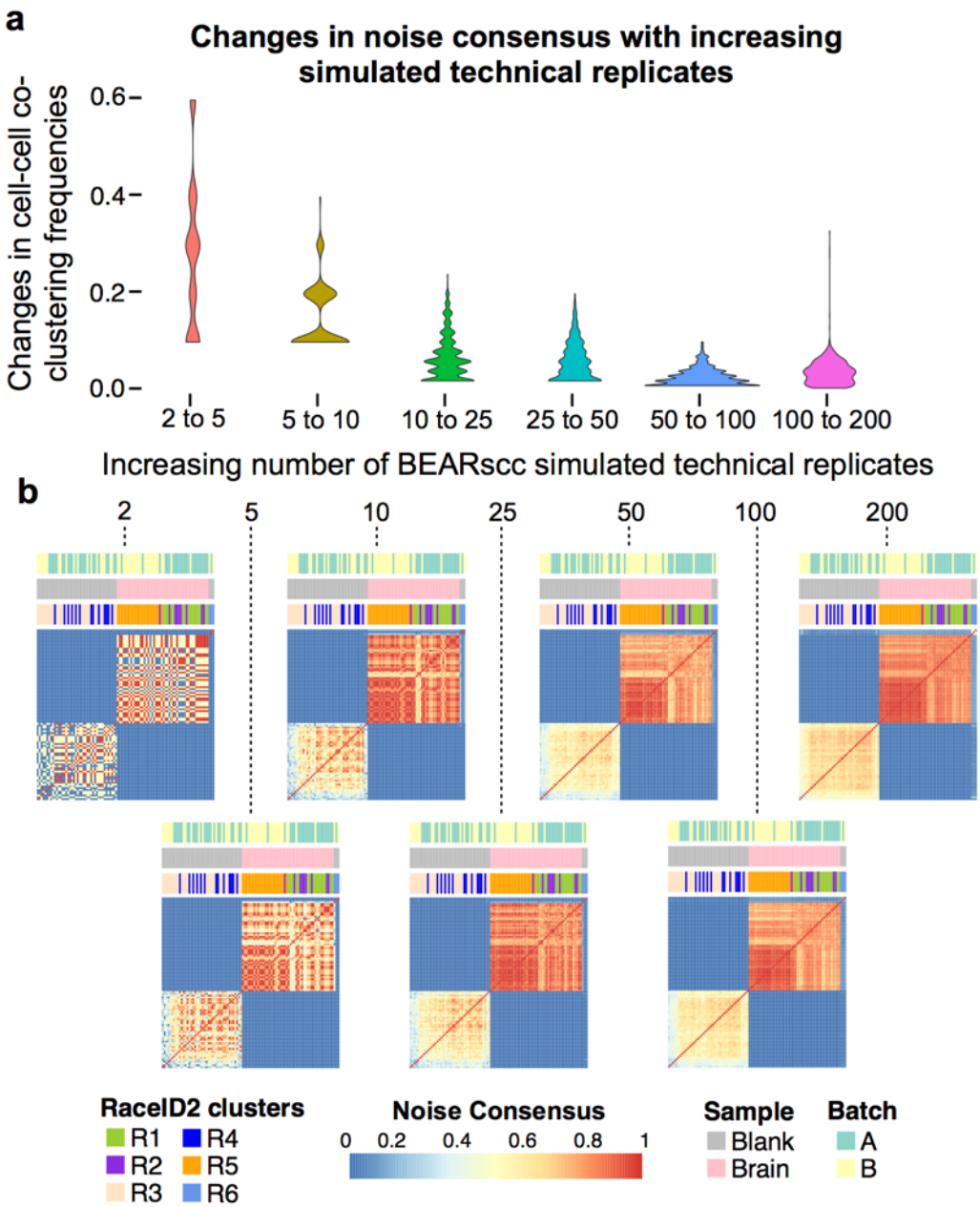
Supplementary Figure 2 BEARscc applied to the brain-and-blanks control experiment in combination with BackSPIN **(a)**, RaceID2 **(b)** and SC3 **(c)**. Top: Separation of clusters by tissue and batch by each algorithm. RaceID2 and SC3 clusters are visibly confounded by batch. Bottom: BEARscc noise consensus matrices for each algorithm. Color annotations above the noise consensus matrices denote the cluster assignment by the respective algorithm, batch, BEARscc noise consensus clusters and sample group, respecitvely.



Supplementary Figure 3 BEARscc identifies robust cells and clusters in murine intestinal data clustered by RaceID2**. a**, Jitterplot displays the per-cell scores relative to the published RaceID2 clustering. **b**, noise consensus matrix for murine intestinal cells clustered with RaceID2. Above heatmap: published (top) and BEARscc-derived consensus clustering (bottom).



Supplementary Figure 4 BEARscc correctly detects that separation of "stem-like" cell clusters R1 and R2 is based on weak expression differences. (**a**) Heatmap of expression of genes characteristic of clusters R1 and R2, and (**b**) clusters R1 and R5, as described by Grün et al. Columns are ordered by library size per cell, rows sorted by significance of expression fold-change between clusters. Boxplots on the left denote the significance of difference in expression between the two clusters (Wilcoxon rank-sum test). Red denotes the observed values, whereas simulated technical replicates are shown in gray. Solid line denotes Bonferroni-corrected significance threshold.



Supplementary Figure 5 The effect of the number of perturbations on cell-cell consensus clustering becomes negligible after 50 perturbations. BEARscc was applied to RaceID2 clustering of brain whole tissue data and blank samples with increasing numbers of simulated technical replicates. **a**, A violin plot illustrating the distribution of element by element changes in the noise consensus matrix as the number of simulated technical replicates increases from 2 to 5 up to 200. **b**, Heatmaps display the noise consensus matrix calculated by BEARscc for each number of simulated technical replicates.