Supplementary material to PsiNorm: a scalable normalization for single-cell RNA-seq data

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1 The Zipf's law and its relation to Pareto

The Zipf's power-law is a power-law discrete distribution based on ranks whose probability mass function is given by:

$$f(k; x, I) = \frac{1/k^s}{H(I, s)} = C_z k^{-s}$$

where I is the number of elements, k the vector of their ranks and s the coefficient characterizing the distribution. $H(I, s)$ is the generalized harmonic series and C_z is equal to $1/H(I, s)$.

The estimation of s is not available in closed form and it is necessary to estimate it through recursive optimization methods. We used the *stats4::mle* R function to compute the estimate of s as the value that maximizes the likelihood function. We used Nelder and Mead (1965) optimization method (the default of *optim* function) which uses the concept of simplex to approximates a local optimum.

Both Pareto and Zipf distributions are simple power laws with negative exponent and Zipf can be derived from the Pareto distribution if X is a Pareto random variables and its values are binned into I ranks.

Specifically, the Pareto's density function $f(x)$ is a power-law:

$$f(x) = \alpha m^{\alpha} x^{-(\alpha+1)} = C_p x^{-\beta}$$

where $C_p = \alpha m^{\alpha}$ and the parameter of a generic power-law β is equal to $\alpha + 1$.

Then, the mean of the k-th random variable X distributed as Zipf is equal to $E[X_k] \sim C_1 \times k^{-s}$ with the meaning that there are k variables with the expected value higher than

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this quantity. We obtain that $Pr[X \geq C_1 \times k^{-s}] = C_2 \times k$ and given $y = k^{-s}$ and deriving the distribution function the result is $P[X = y] \sim y^{-1+(1/s)} = y^{-\beta}$. So from the relationships between α and β and between β and s we obtain:

$$\beta = \alpha + 1 = 1 + 1/s \Rightarrow \alpha = 1/s$$

2 Banchmarked methods

Count per million (CPM). This method simply divides read counts by the sequencing depth defined as the sum of the expression of the genes per cell. Each count is then multiplied by a million to make normalized count not too much compressed. Usually, the base 2 logarithm of the normalized pseudo-count is taken, defining the *logCPM* values:

$$\tilde{x}_{ij} = log_2\Big(\frac{x_{ij} \times 10^6}{N_j} + 1\Big)$$

with $N_j = \sum_i x_{ij}$ the sum of the counts of cell j.

Centered Log-Ratio (CLR). CLR is similar to logCPM with the difference that it divides pseudo-counts by the geometric mean of each cell. Given gm_{x_j+1} the geometric mean of the j-th cell:

$$gm_{x_j+1} = \Big(\prod_{i=1}^{n}(x_{ij}+1)\Big)^{\frac{1}{n}}$$

the normalized counts are:

$$\tilde{x}_{ij} = log\Big(\frac{x_{ij}}{gm_{x_j+1}} + 1\Big)$$

scran. scran is based on a cell pooling strategy. Given the global reference $\overline{x}$ defined as:

$$\overline{x} = \frac{1}{J}\sum_j x_{ij}, \quad i = 1, ..., n, \quad j = 1, ..., J$$

and k overlapping groups of cells, scran estimates the size factor SF_{p_k} of each pool under the assumption that every SF_{p_k} is a linear combination of the size-factors of the cells that belong to the pool:

$$\forall\ pool_k\ : \sum_{j \in p_k} x_{ij} = \Big[x_{1p_k}, ..., x_{np_k}\Big]$$

$$SF_{p_k} = Median\Big(\frac{x_{1p_k}}{\overline{x}_1}, ..., \frac{x_{np_k}}{\overline{x}_n}\Big) = \sum_{j \in p_k} SF_j$$

Solving the equations, we obtain size factors for each cell and define the normalized values as:

$$\tilde{x}_{ij} = log\Big(\frac{x_{ij}}{SF_j} + 1\Big)$$

DESeq2 Deseq2 uses as reference the geometric means of the of gene across cells. For every gene i:

$$gm_{x_i} = \Big(\prod_{j=1}^{J} x_{ij}\Big)^{\frac{1}{J}}$$

Then each count is divided by its geometric mean and the median of these ratios is the size factor for the sample j.

$$SF_j = Median\Big(\frac{x_{1j}}{gm_{x_1}}, ..., \frac{x_{nj}}{gm_{x_n}}\Big)$$

The normalized counts are obtained by taking the log of the ratio of each counts and its size factor:

$$\tilde{x}_{ij} = log_2\Big(\frac{x_{ij}}{SF_j} + 1\Big)$$

Trimmed Mean of M-values (TMM). TMM (Robinson and Oshlack, 2010) defines the log-fold-changes (M) and absolute expression levels (A) between each cell and a reference (by default the cell whose upper quartile is closest to the mean upper quartiles across cells):

$$\begin{aligned} M_{ij}^{(r)} &= log_2\Big(\frac{x_{ij}/N_j}{x_{ir}/N_r}\Big) \\ A_{ij}^{(r)} &= \frac{1}{2} log_2(x_{ij}/N_j * x_{ir}/N_r) \end{aligned}$$

to apply a trimming procedure. By default the method trims the 30% of highest and lowest values for M_{ij} and 5% of highest and lowest values for A_{ij}. After the trimming, the mean of M_{ij} weighted by the inverse of the approximate asymptotic variances is used to normalize the counts:

$$\begin{aligned} log_2(SF_j^{(r)}) &= \frac{\sum_{i \in I^*} M_{ij}^{(r)} w_{ij}^{(r)}}{\sum_{i \in I^*} w_{ij}^{(r)}} \\ w_{ij}^{(r)} &= \frac{N_j - x_{ij}}{N_j x_{ij}} + \frac{N_r - x_{ir}}{N_r x_{ir}} \\ \tilde{x}_{ij} &= log_2\Big(\frac{x_{ij}}{SF_j N_j} + 1\Big) \end{aligned}$$

where I^* is the set of genes with valid M_{ij} and A_{ij} values.

Linnorm. **Linnorm** (Yip *et al.*, 2017) filter genes according to their sparseness, variability and skewness in order to identify a set of stable genes. Then, given $R_{ij} = \frac{x_{ij}}{N_j}$ it defines the log of the normalized pseudo-counts as follow:

$$T_{ij} = ln(\lambda R_{ij} + 1)$$

The purpose is to identify the λ (dataset-specific) that minimize the deviation from homoscedasticity and normality:

$$F(\lambda) = V(\lambda)^2 + S(\lambda)^2$$
$$\lambda = argmin(F(\lambda))$$

where $V(\lambda)$ represents the deviation of T_{ij} from homoscedasticity and $S(\lambda)$ the deviation from the skewness of the dataset. Once $\hat{\lambda}$ has been obtained, *Linnorm* uses the quantities $G_{ij} = ln(\hat{\lambda} R_{ij})$ to define n regression models, $g_i = m_j x_{ij} + c_j$, where g_i is the mean expression and x_{ij} the sample's expression. Model parameters, m and c, are updated with the equations $m^{updated} = \mu(m-1)+1$ and $c^{updated} = c \times \mu$ with μ set by default to 0.5 which provides a moderate level of normalization strength. Finally, given $B_{ij} = exp(m_j^{updated} G_{ij} + c_j^{updated})$ the counts are normalized:

$$\tilde{x}_{ij} = ln(B_{ij}+1)$$

sctransform. **sctransform** is based on a regression model per gene with negative binomial error distribution and logarithmic link function (Hafemeister and Satija, 2019). For a given cell j and gene i it can define the expected counts and the expected standard deviation as follow:

$$log(\mu_{ij}) = \beta_{0_i} + \beta_{1_i} log_{10} N_j$$
$$\sigma_{ij} = \sqrt{\mu_{ij} + \frac{\mu_{ij}^2}{\theta_i}}$$

where β_{0_i} and β_{1_i} and the dispersion parameter θ have to be estimated. To avoid overfitting, SCT exploits the trend of the estimates versus gene mean to perform independent regularizations for all parameters. The regularized parameters are used to define the normalized counts as the Pearson residuals of the model:

$$\tilde{x}_{ij} = \frac{x_{ij} - \mu ij}{\sigma_{ij}}$$

where μ_{ij} is the expected count of gene i in cell j in the regularized negative binomial regression model, and σ_{ij} is the expected standard deviation.

3 Supplementary Tables

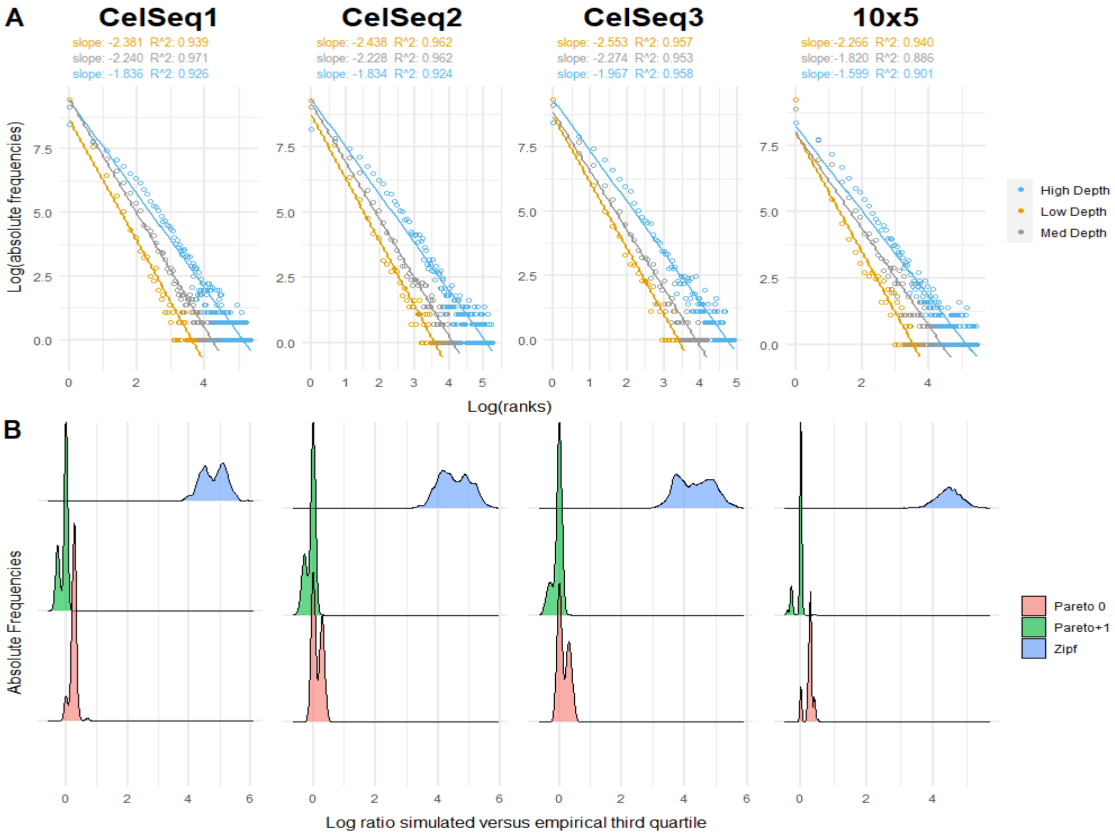
Supplementary Table 1: Description of the datasets used to compare and evaluate PsiNorm normalization performances.

Dataset name	N. genes	N. cells	N. clusters	% nulls	Technology	Sample type	Reference
10x	16468	902	3	.45	10x	Cell mixture	mixology Tian et al. (2019)
CELSeq	19759	274	3	.64	celseq	Cell mixture	mixology Tian et al. (2019)
DropSeq	14947	225	3	.62	dropseq	Cell mixture	mixology Tian et al. (2019)
CELSeq51	15564	297	5	.61	celseq	Cell mixture	mixology Tian et al. (2019)
CELSeq52	14078	307	5	.60	celseq	Cell mixture	mixology Tian et al. (2019)
CELSeq53	13426	305	5	.64	celseq	Cell mixture	mixology Tian et al. (2019)
10x5	11786	3918	5	.63	10x	Cell mixture	mixology Tian et al. (2019)
csmart	17998	500	14	.53	smart	cells	BICCN Zeng Yao et al (2020)
nsmart	17902	500	17	.73	smart	nucleus	BICCN Zeng Yao et al (2020)
cV2	15784	500	17	.73	10x	cells	BICCN Zeng Yao et al (2020)
cV3	16837	500	17	.61	10x	cells	BICCN Zeng Yao et al (2020)
nV2	14791	500	14	.89	10x	nucleus	BICCN Zeng Yao et al (2020)
nV3	15889	500	15	.80	10x	nucleus	BICCN Zeng Yao et al (2020)

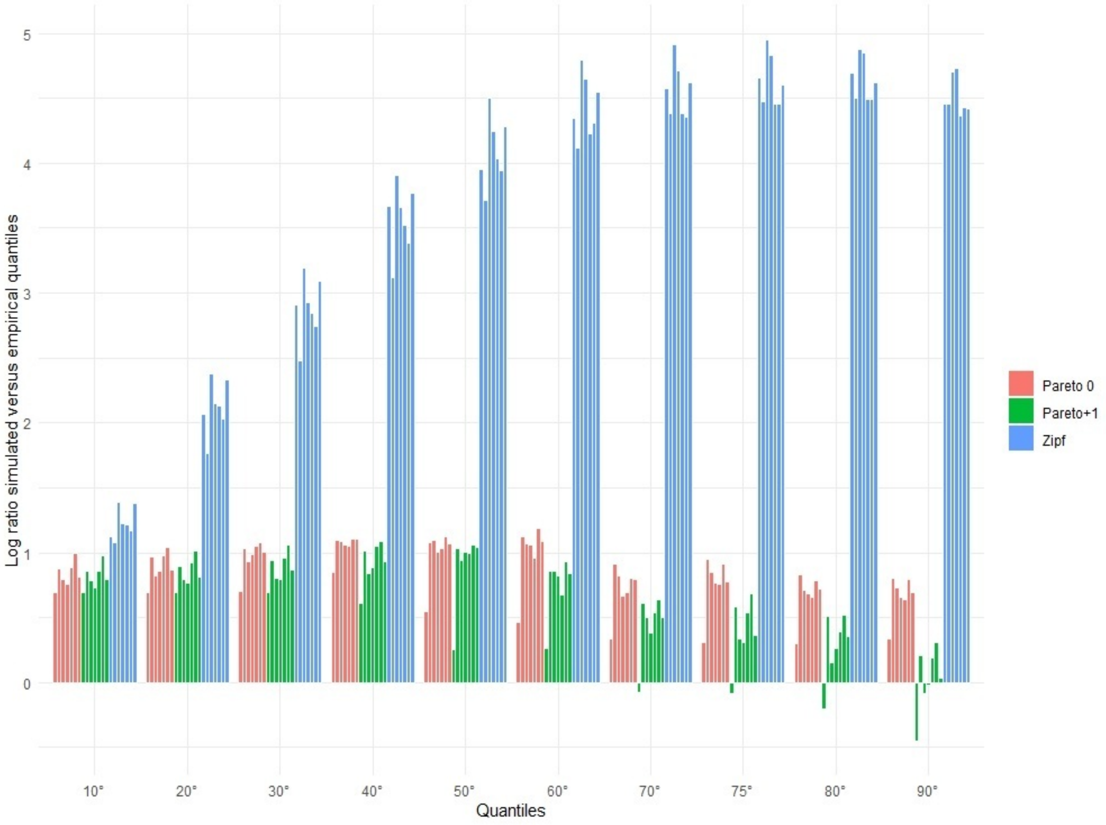
Supplementary Table 2: Normalization evaluation using median values of selected metrics. See Table 1 of the main text for average values.

	Median	Median	Median correlation	Median within	Median between
	ARI	silhouette	PCA-depth	concordance	concordance
sctransform	0.796	**0.248**	0.313	**0.582**	0.256
Linnorm	0.888	0.244	0.310	0.481	0.267
PsiNorm	0.879	0.224	0.421	0.532	0.247
Scran	**0.922**	0.241	0.345	0.492	0.211
TMM	0.454	0.232	0.515	0.511	**0.281**
logCPM	0.703	0.176	0.426	0.504	0.219
DESeq	0.689	0.225	**0.303**	0.504	0.244
CLR	0.621	0.187	0.783	0.490	0.263

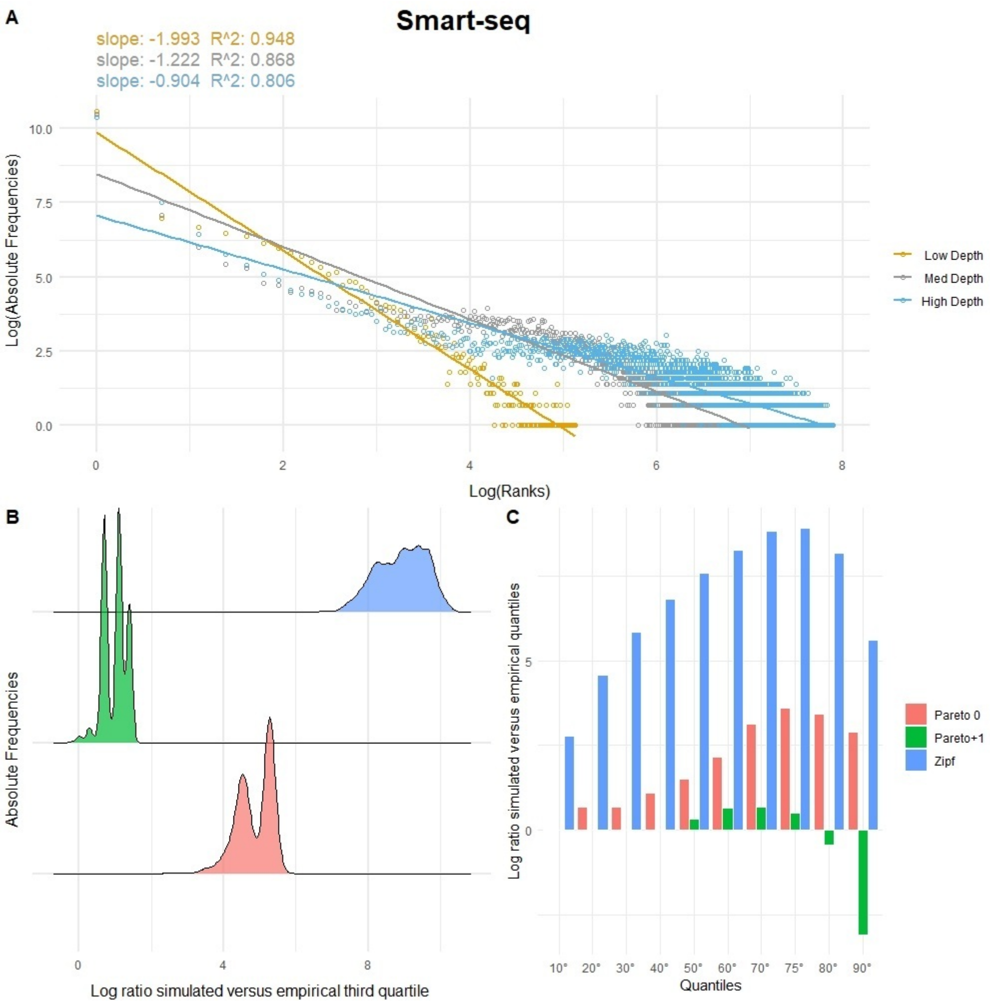
4 Supplementary Figures



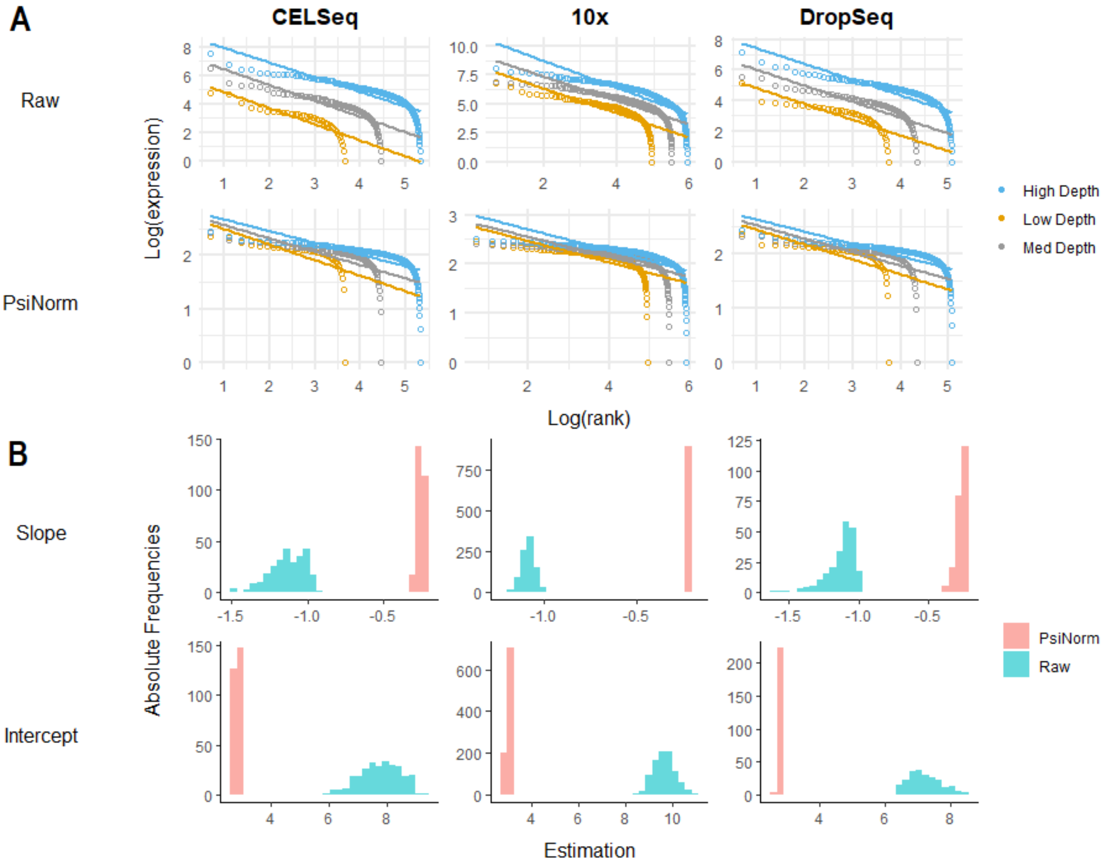
Supplementary Figure 1: Goodness of fit. **Panel A.** Log-frequency vs log rank plot of three cells representative of the minimum, median and maximum depth per technology. The rank is based on the unique expression values from the lowest to the highest. Each dot in the graph represents more than one gene, namely all the genes that share the same expression value in that cell. Linear fit is reported along with least-squares estimates of the slopes and R^2 values of the linear fit. **Panel B.** Distribution of the log ratios between simulated and empirical third quartiles per cell across different technologies. Supplementary Figure S2 shows the same quantity for other quantiles. The figure shows that the Zipf simulated quantiles are far from the empirical ones, while the Pareto distribution (especially when applied to $x + 1$) provides a much better fit given that values are closer to zero.



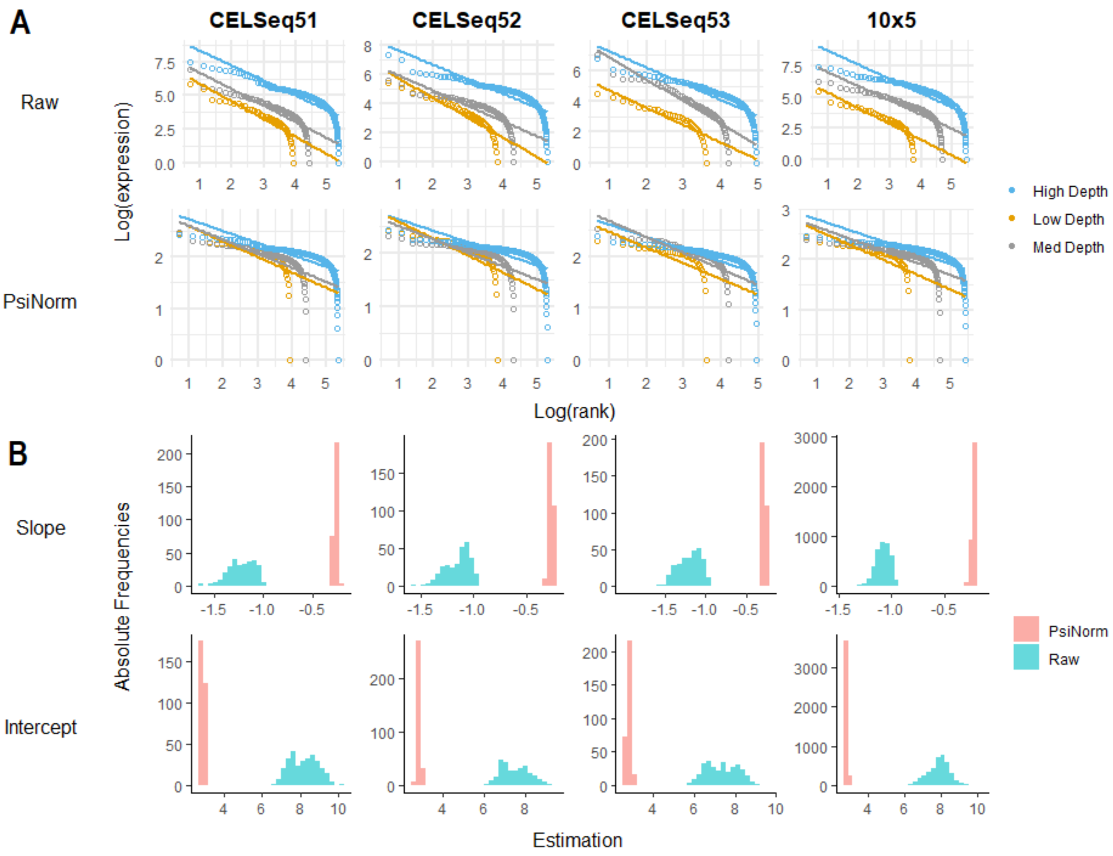
Supplementary Figure 2: Goodness of fit. Average log ratios between observed and expected quantiles across cells for different technologies and models. Colors represent different models, while bars of the same colors represent from the left to the right 10x, 10x5, celseq2, celseq51, celseq52, celseq53 and dropseq datasets.



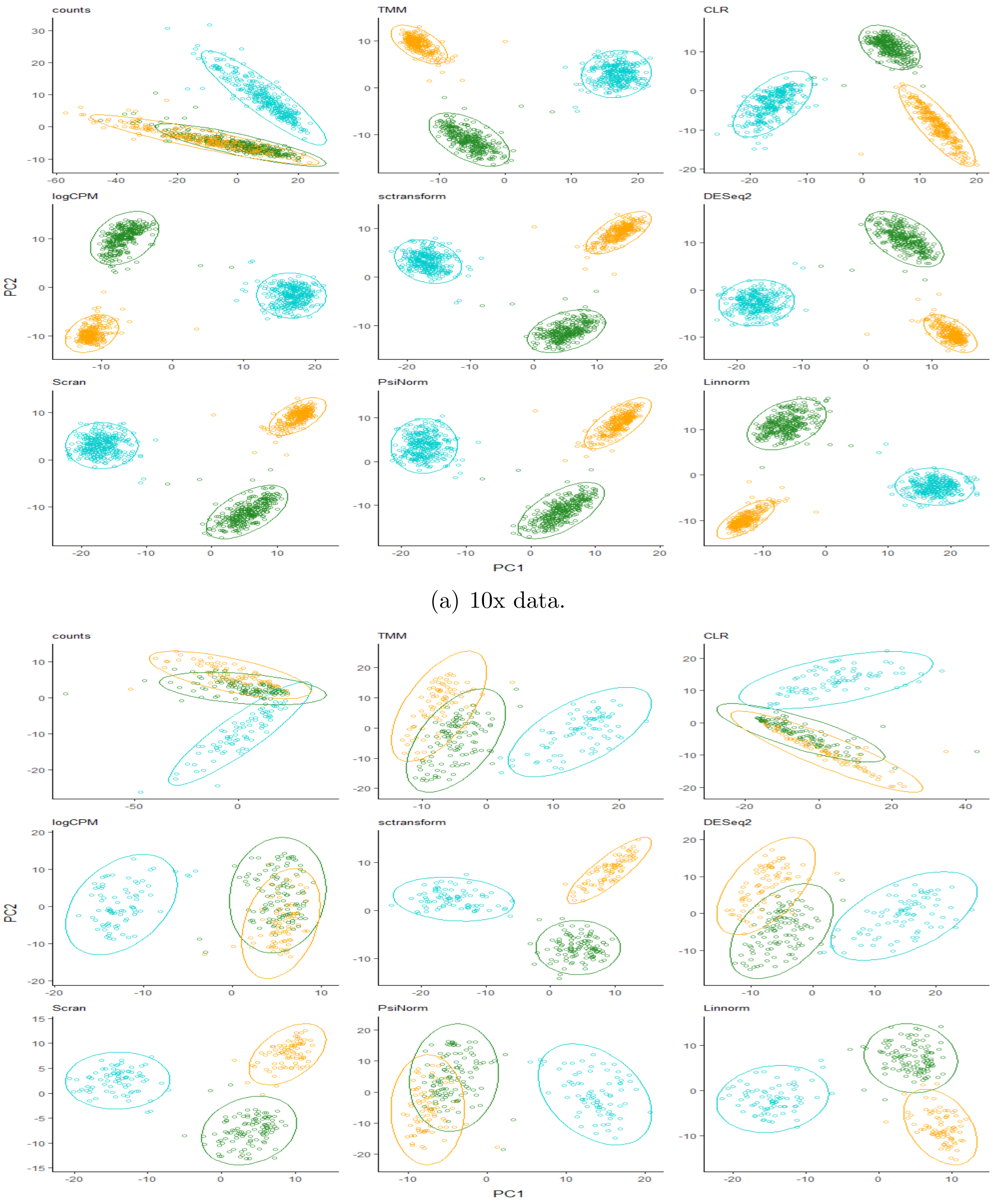
Supplementary Figure 3: Goodness of fit for Smartseq data. **Panel A.** Log-frequency vs log rank plot of three cells representative of the minimum, median and maximum depth per technology. The rank is based on the unique expression values from the lowest to the highest. Each dot in the plot represents more than one gene, namely all the genes that share the same expression value in that cell. Linear fit is reported along with least-squares estimates of the slopes and R^2 values of the linear fit. **Panel B.** Distribution of the log ratios between simulated and empirical third quartiles per cell. **Panel C.** Average log ratios between observed and expected quantiles across cells. Colors represent different models.



Supplementary Figure 4: Pareto normalization. **Panel A.** The log expression versus the log rank (ordered from the highest to the lowest) of three cells representative of low, moderate and high coverage, is reported for raw and Pareto normalized data. The linear fit is reported for each cell. **Panel B.** The density distributions (across all cells per technology) of the linear fit estimates (slopes and intercepts) of raw and normalized data.



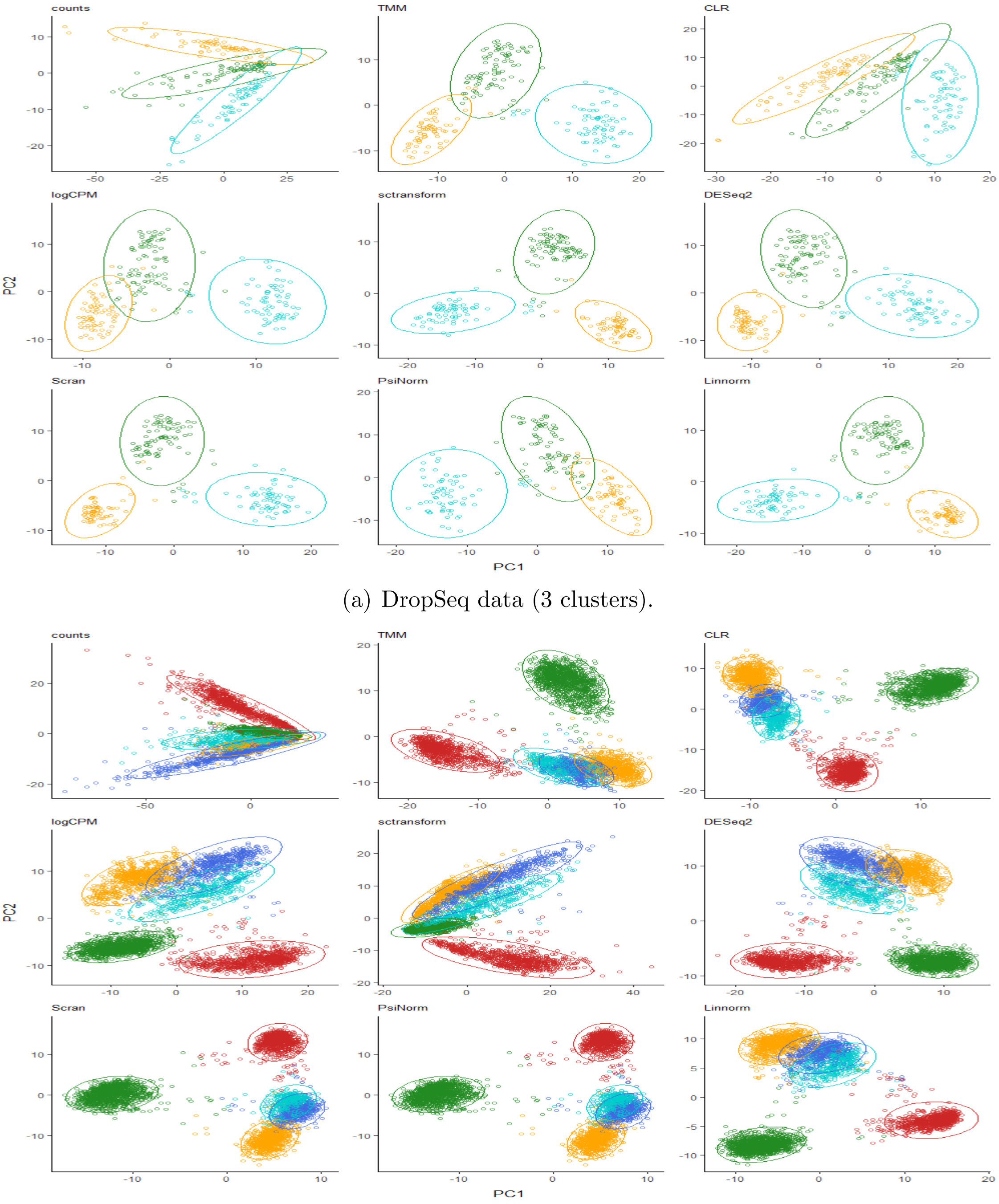
Supplementary Figure 5: Pareto normalization. **Panel A.** The log expression versus log rank (ordered from the highest to the lowest) of three cells representative of low, moderate and high coverage, is reported for raw and Pareto normalized data. The linear fit is reported for each cell. **Panel B.** The density distributions (across all cells per technology) of the linear fit estimates (slopes and intercepts) of raw and normalized data.



(a) 10x data.

(b) CELSeq data.

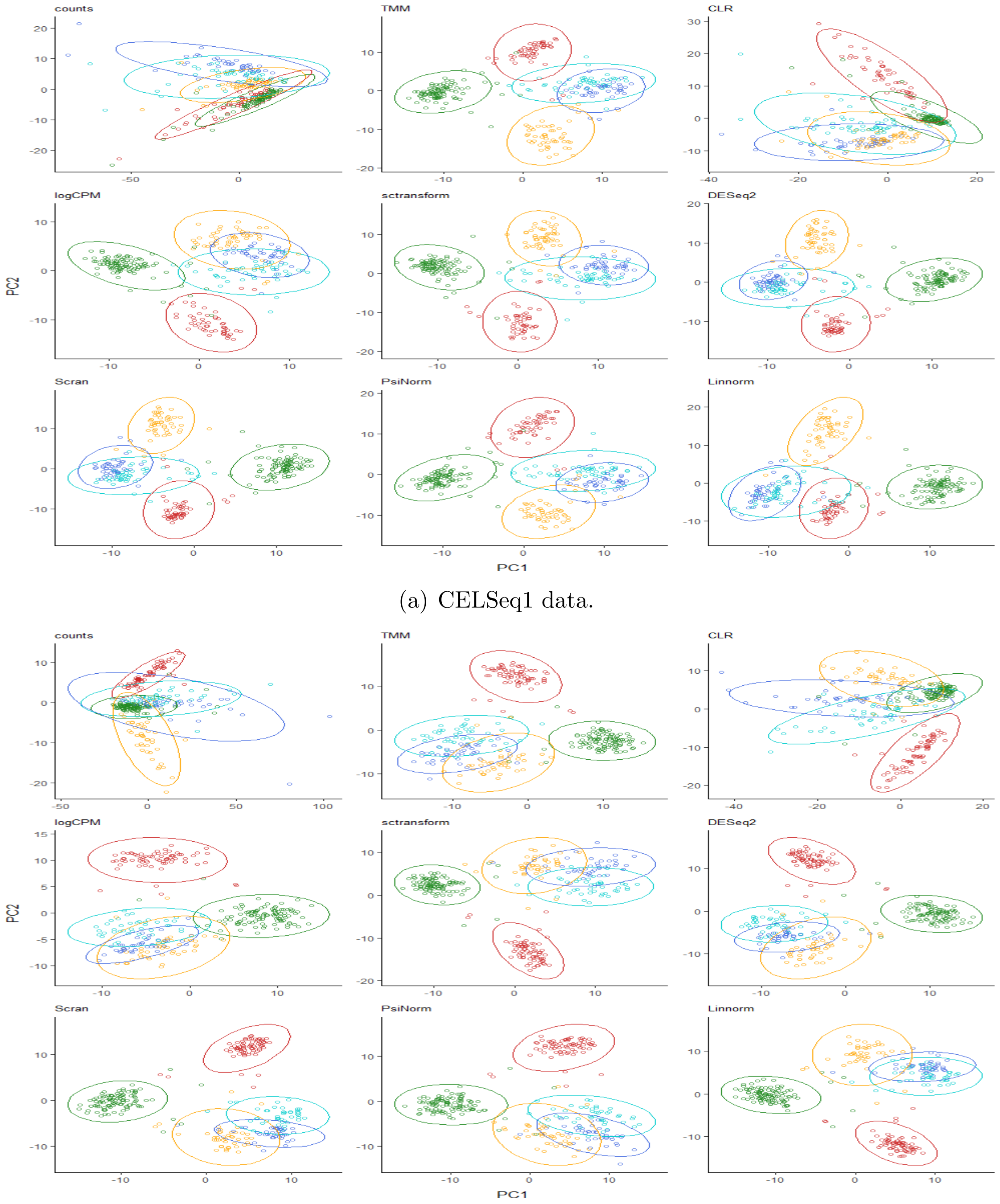
Supplementary Figure 6: PCA plot of the data with 3 cell types (highlighted with different colours and by data ellipses using multivariate t-distribution)



(a) DropSeq data (3 clusters).

(b) 10x5 data (5 clusters).

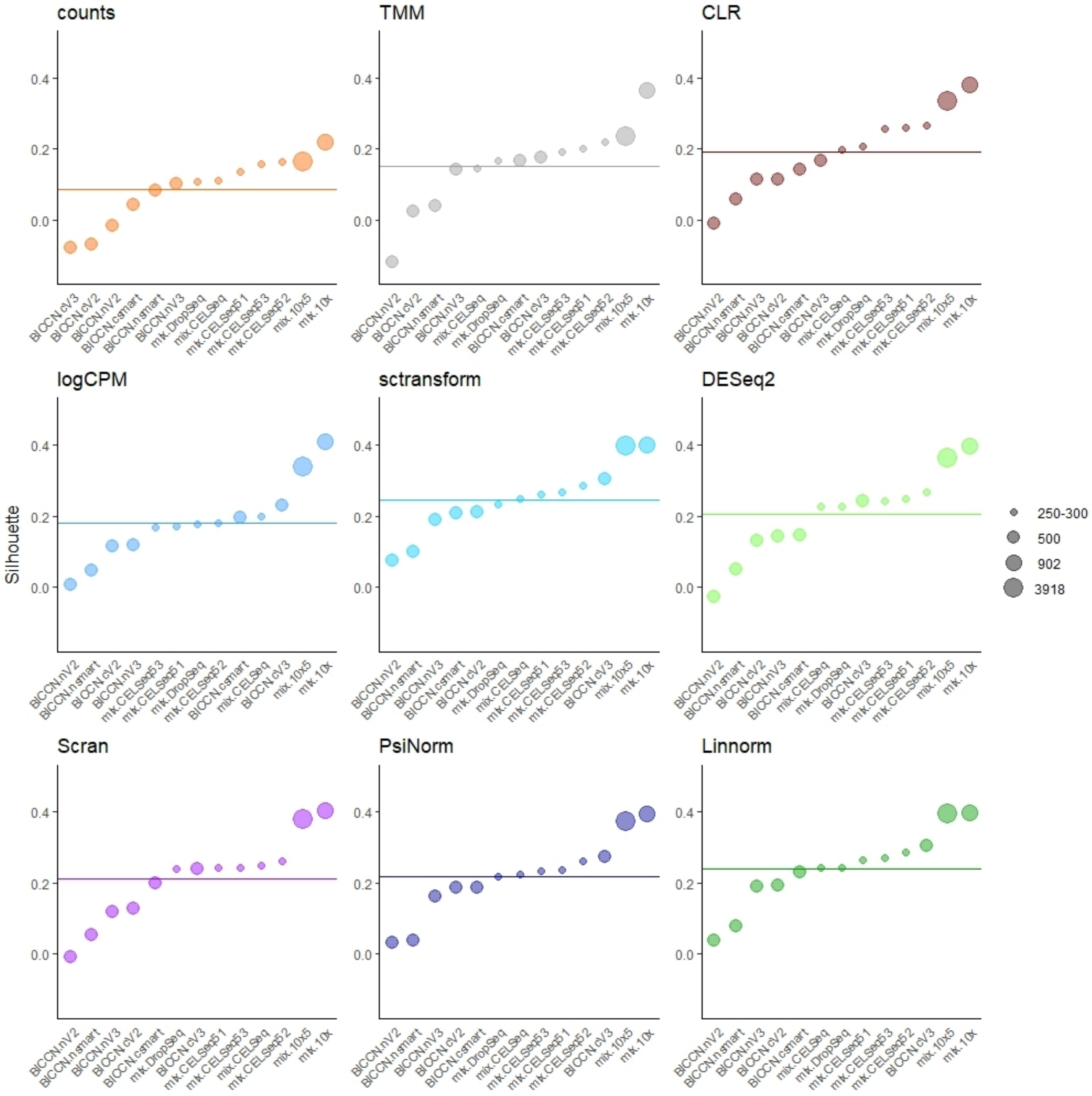
Supplementary Figure 7: PCA plot of the data with 3 and 5 cell types (highlighted with different colours and by data ellipses using multivariate t-distribution)



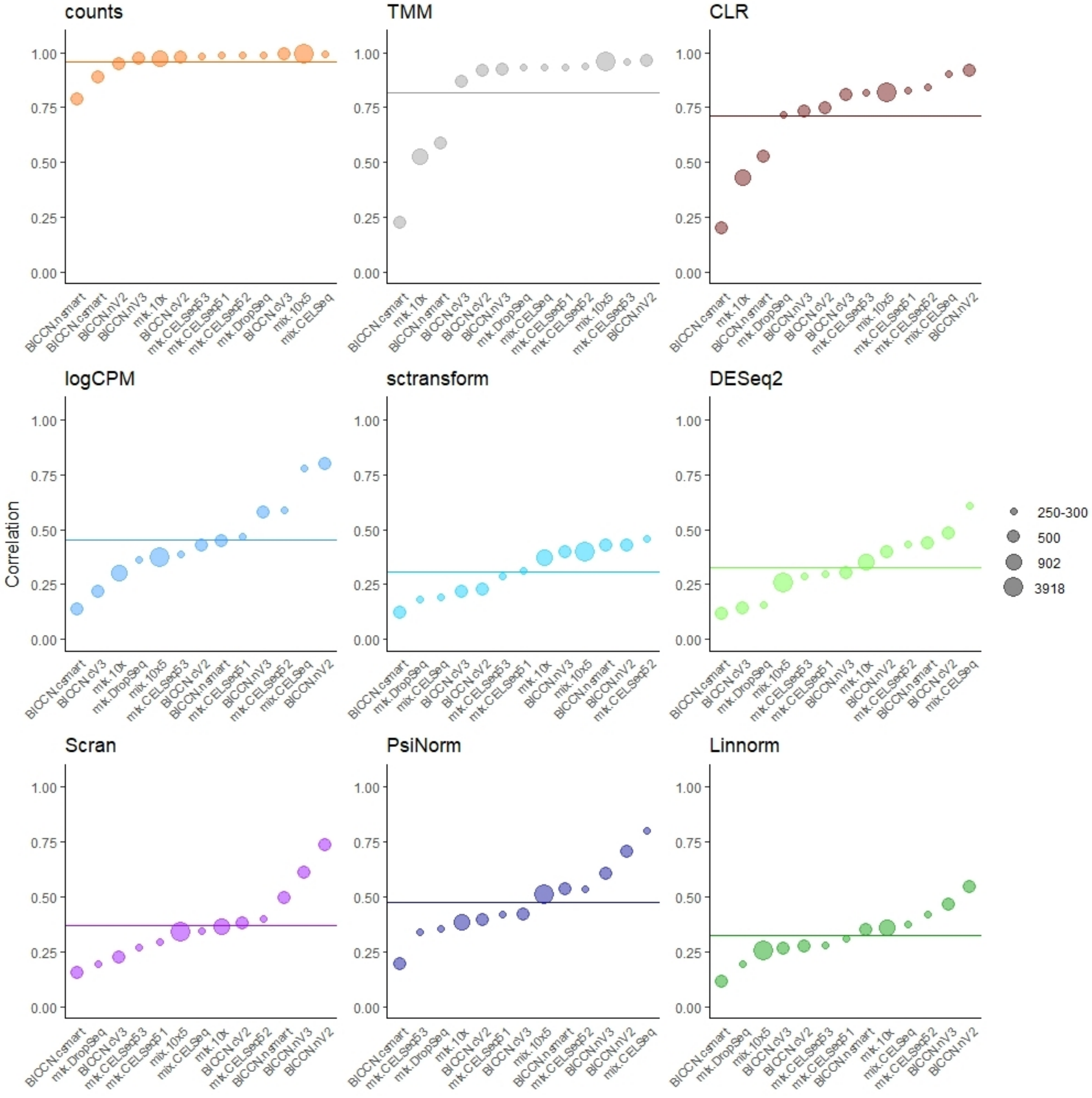
(a) CELSeq1 data.

(b) CELSeq2 data.

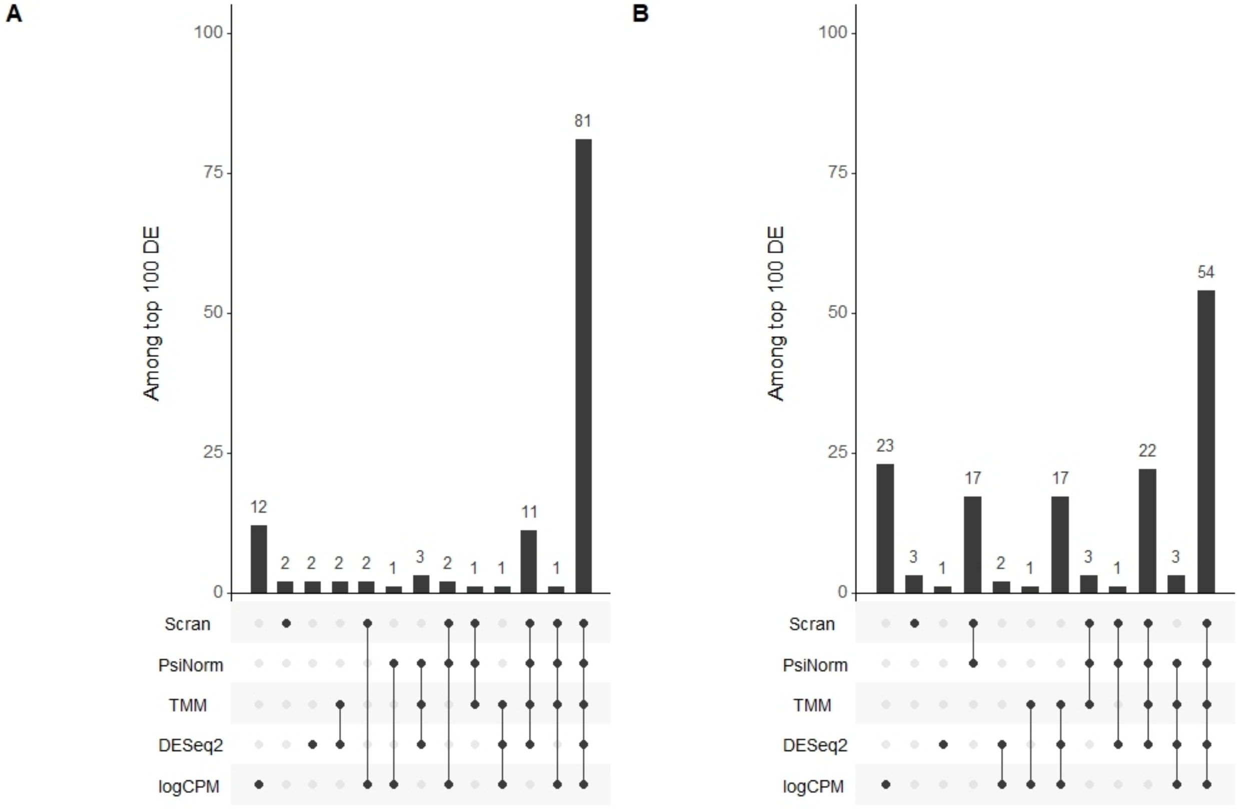
Supplementary Figure 8: PCA plot of the data with 5 cell types (highlighted with different colours and by data ellipses using multivariate t-distribution)



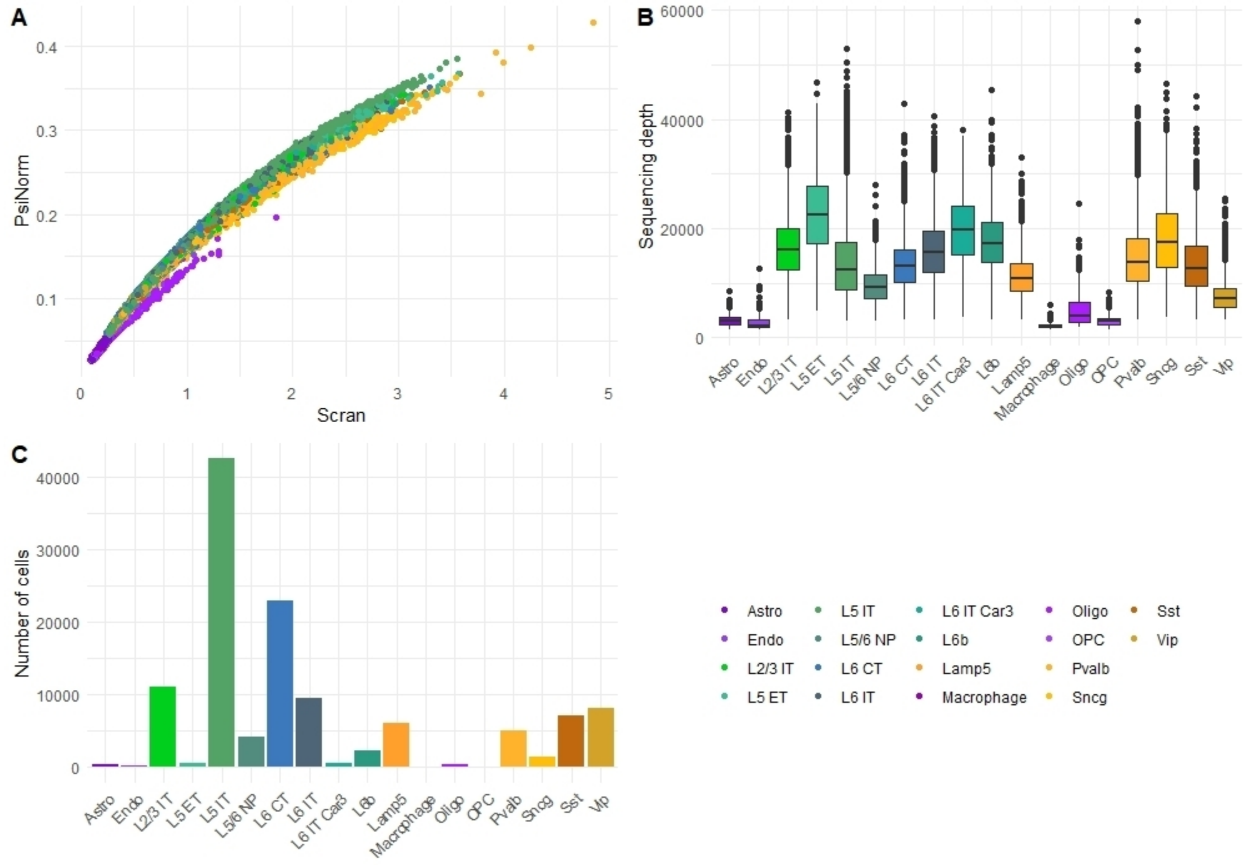
Supplementary Figure 9: Silhouette index across different dataset and different normalization methods. Datasets are sorted by the silhouette index. The dot size is proportional to the dimension of the datasets in terms of number of cells.



Supplementary Figure 10: The maximum correlation index between PC1 and PC2 and cell sequencing depths is reported for each dataset. Datasets are sorted by the correlation index. The dot size is proportional to the dimension of the datasets in terms of number of cells.



Supplementary Figure 11: Upset plot obtained comparing the top 100 differentially expressed genes (obtained using *edgeR* package) with the selected normalization methods. scran with default setting and with clustering option give the same top 100 differentially expressed genes, the second is not reported in the Figure. **Panel A** Pvalb vs Sst cell types comparison **Panel B** Astro vs Oligo cell types comparison



Supplementary Figure 12: **Panel A** Scatterplot comparing scran and *PsiNorm* scaling factors. **Panel B** Boxplot of the distribution of the sequencing depths for each cell type. **Panel C** Barplot of the frequency of cells within each cell types. Colors represent different population cell types.

References

Hafemeister, C. and Satija, R. (2019). Normalization and variance stabilization of single-cell rna-seq data using regularized negative binomial regression. *Genome biology*, **20**(1), 1–15.

Robinson, M. D. and Oshlack, A. (2010). A scaling normalization method for differential expression analysis of rna-seq data. *Genome biology*, **11**(3), 1–9.

Yip, S. H., Wang, P., Kocher, J.-P. A., Sham, P. C., and Wang, J. (2017). Linnorm: improved statistical analysis for single cell rna-seq expression data. *Nucleic acids research*, **45**(22), e179–e179.