GigaScience

rCASC: reproducible Classification Analysis of Single Cell sequencing data

--Manuscript Draft--

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Full Title:	rCASC: reproducible Classification Analysis of Single Cell sequencing data
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Abstract:	Background Single-cell RNA sequencing is an essential tool to investigate cellular heterogeneity, and to highlight cell sub-population specific signatures. Single-cell sequencing applications are now spreading from the most conventional RNAseq to epigenomics, e.g. ATAC-seq. Single-cell sequencing led to the development of a large variety of algorithms and associated tools. However, to the best of our knowledge, there are few computational workflows providing analysis flexibility and achieving at the same time functional (i.e. information about the data and the utilized tools are saved in terms of meta-data) and computational reproducibility (i.e. real image of the computational environment used to generate the data is stored) through a user-friendly environment. Findings rCASC is a modular workflow providing integrated analysis environment (from counts generation to cell subpopulation identification) exploiting docker containerization to achieve both functional and computational reproducibility in data analysis. Hence, rCASC provides preprocessing tools to remove low quality cells and/or specific bias, e.g. cell cycle. Subpopulations discovery can be instead achieved using different clustering techniques based on different distance metrics. Quality of clusters is then estimated through a new metric namely Cell Stability Score (CSS), which describes the stability of a cell in a cluster as consequence of a perturbation induced by removing a random set of cells from the overall cells' population. Our experiments highlight that CSS provides better cluster-robustness information than silhouette metric. Moreover, rCASC provides tools for the identification of clusters-specific gene-signature. Conclusions rCASC is a modular workflow with valuable new features that could help researchers in defining cells subpopulations and in detecting subpopulation specific markers. It exploits docker framework to make easier its installation and to achieve a computation reproducible analysis. Moreover, a Java Graphical User Interface (GUI), is provided in rCASC to make friendly the use of the tool even for users without computational skills in R.
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Response to Reviewers:	Rebuttal letter for the paper: rCASC: reproducible Classification Analysis of Single Cell sequencing data. Luca Alessandrì, Francesca Cordero, Marco Beccuti, Maddalena Arigoni, Martina Olivero, Greta Romano, Sergio Rabellino,Nicola Licheri, Gennaro De Libero, Luigia Pace and Raffaele A Calogero Dear Editor, First of all, we wish to thank the reviewers for their valuable comments and useful suggestions which helped us to substantially improve the paper and its associated tool. Hereafter we report our answers to the reviews' comments. Reviewer #1 First, I would like to thank the authors for the hard and great work they did to address the different major aspect previously mentioned. Notably, they evaluated the time complexity of random, artificially created datasets, using 160 permutations, using 6 different sizes for both features (800 to 10000) and number of cells (from 400 to 5000). They also rewritten several sections of the manuscript such as the CSS part, and corrected different concerning aspects (such as the "supervised clustering" designation). They also simplified their pipeline and added a "mini" option to be able to download only a few Docker containers on all the available containers of rCASC which are enough to provide basic functionalities to handle single-cells. Finally, they investigated the relationship between stability and cluster significant with additional experiments. In the other hand, I am a little bit disappointed by the performance of rCASC in term of scaling-up. Using rCASC, it seems difficult to process and cluster more than 5K cells with a reasonable machine, such as a personal computer of a cluster node (with RAM up to 64GiG), and in a reasonable time (less than 24-48h). Unfortunately, I was expecting this result because rCASC uses a lot of tools that at some point require the computation of a step having a polynomial complexity. For example, SIMLR require the computation of a cell-cell distance matrix. Increasing the number of cells gives rapidly a matrix that either takes a very long time to compute /and/or cannot be loaded in the RAM of the computer. Being able to process a larger number of cells is I think a very important aspect of any new single-cell bioinformatic pipeline because A) the technology is growing rapidly and we can expect a larger amount of cells to be processed in the near future, and B) more and more single cell datasets are available leading to meta-analyses combining multiple single-cell datasets. My first comment is: is there any settings of rCASC that can be used to process a dataset larger than 5K cells? Ideally 100K (See for example Scanpy pipeline that can process up to 1M) would be a good number. However, I think that at least 10K cells is a minimum. For example, it seems that Griph is a method the less greedy in term of resources used. Then, maybe a specific set of settings designated for "very_large_dataset" processing can be used with Griph to be able to compute larger dataset? In that case, not all the options of rCASC need to be used (especially the ones using a lot of resources). Is it possible to adapt the stability metric to such datasets? However, if the authors think that clustering datasets larger than 5K cells is

out of scope of this study what would be the reason to justify the non-scalability of the method? I agree that rCASC allows a very rigorous and complete analysis of a smaller dataset but is the processing of 1 to 5K cells enough considering the single-cell RNA-Seq field evolution (it is an open question)?

Answer #1:
We incorporated in rCASC the clustering tools scanpy and griph as required by the reviewer. Moreover, we tested the performance of rCASC using Seurat, griph, and scanpy tools considering 10K, 35K, 68K and 101K cells. The analysis was executed on an SGI server (10 x CPU E5-4650 2.4GHz (16 cores), 1TB RAM, 30 TB SATA raid disk) allocating 40 threads for each analysis.
The results of this comparison highlight that griph works slightly faster than Seurat tool. However, both these tools do not scale well when more than 68K cells are analyzed. Differently scanpy shows the best performance up to 101K cells.
Hence, figure 7 was redraw to report the new results, and its caption “Scalability analysis of clustering tools implemented in rCASC. A) Time required to perform 160 permutations as function of increasing number of cells on a set of 27998 genes. B) Time required to perform 160 permutations as function of increasing number of genes on a set of 800 cells.” was updated in the following way:
“Scalability analysis of the clustering tools implemented in rCASC. A) Time required to perform 160 permutations as function of increasing number of cells on approximately 20,000 genes. Left panel: SIMLR, tSne, Seurat and griph clustering up to 5,000 cells was executed on a SeqBox [7] (1 x CPU i7-6770HQ 3.5 GHz (8 cores), 32 GB RAM, 1TB SSD). Right panel: Seurat, griph and scanpy analyses were extended until 101,000 cells using an SGI server (10 x CPU E5-4650 2.4GHz (16 cores), 1TB RAM, 30 TB SATA raid disk). B) Time required to perform 160 permutations as function of increasing number of genes on a set of 800 cells, analysis performed on a SeqBox.”

Moreover, Scalability section in the main manuscript “To estimate the scalability of rCASC clustering we used the GSE106264 dataset made of 10035 cells and published by Pace and coworkers in 2018 [21]. We randomly sampled the 10035 cells (27998 ENSEMBL GENE IDs) to obtain the following subsets of cells: 400, 600, 800, 1000, 2000, 5000. Starting from the 800 cells set we randomly sampled the genes: 10000, 8000, 6000, 4000, 2000, 1000, 800. We run SIMLR, tSne, griph and Seurat using 160 permutation within SeqBox hardware [7]: Intel i7 3.5GHz (4 cores), 32 GB RAM and 500 GB SSD disk. SIMLR resulted to be the slowest and, given the above hardware implementation, it cannot allocate for the analysis more than 2000 cells (Figure 7A). All the other tools were able to handle up to 5000 cells within the limit of 32 GB of RAM available in the hardware setting used in this analysis. Computation time was nearly linear for all tools till 1000 cells. Only griph clustering resulted to be nearly insensitive to the increasing number of cells (Figure 7A). The computing time as function of increasing number of genes has a quite limited effect on the overall computing time (Figure 7B).” was updated as follows:
“To estimate the scalability of rCASC clustering we used the GSE106264 dataset made of 10,035 cells and published by Pace and coworkers in 2018 [23] and the 10,000/33,000/68,000 cells PBMC human datasets, available at 10xGenomics repository (www.10xgenomics.com).
We randomly generated from the 10035 cells (27998 ENSEMBL GENE IDs) the following subsets of cells: 400, 600, 800, 1000, 2000, 5000. Moreover, for the subsets with more than 600 cells we randomly sampled the genes: 10000, 8000, 6000, 4000, 2000, 1000, 800. We run SIMLR, tSne, griph and Seurat using 160 permutations within SeqBox hardware [7]: Intel i7 3.5GHz (4 cores), 32 GB RAM and 500 GB SSD disk. SIMLR resulted to be the slowest and, given the above hardware implementation, it cannot allocate for the analysis more than 2000 cells (Figure 7A left panel). All the other tools were able to handle up to 5000 cells within the limit of 32 GB of RAM available in the hardware setting used in this analysis. Computation time was nearly linear for all tools till 1000 cells. Only griph clustering resulted to be nearly insensitive to the increasing number of cells (Figure 7A). We extended, for Seurat, griph and scanpy, the scalability analysis to 10K, 33K, 68K and 101K cells, using 10,000/33,000/68,000 cells from PBMC human datasets, available at 10xGenomics repository (www.10xgenomics.com), and 101,000 cells dataset, made assembling the above mentioned 33,000 and 68,000 PBMC datasets. The analysis was executed on a SGI server (10 x CPU E5-4650 2.4GHz (16 cores), 1TB RAM, 30 TB SATA raid disk) allocating 40 threads for each analysis. Scanpy outperforms the other two methods

	and griph behaves slightly better than Seurat (Figure 7A right panel). The computing time as function of increasing number of genes has a quite limited effect on the overall computing time (Figure 7B).” Finally, the supplementary file was updated adding all the new functionalities implemented as consequence of the reviewer’s requests. Reviewer #2 The authors have now extensively revised the manuscript and added a number of useful functions to the existing rCASC package. By doing so, the authors addressed the majority of my previous comments. However, there remain a few minor comments that should be addressed prior to publication. 1) Relating to the previous major comment 1: The publication associated to the griph package can now be cited as: Serra et al., Self-organization and symmetry breaking in intestinal organoid development. Nature (2019). Answer 1: we updated the reference. 2) Relating to the previous major comment 7: It would be useful to provide more details on which variability measure (variance, squared coefficient of variation or a regression residual) is used to rank genes based on their variability in the topx function while setting type="variance". Answer 2: we updated the description of topx function in the supplementary material specifying that user can decide to use the top expressed/variable genes for clustering. The function **topx** provides the selection of the X top expressed genes given a user defined threshold. Hence, in the supplementary material the following sentences were revised: “For clustering purposes user might decide to use the top expressed/variable genes. The function **topx** provides two options: the selection of the X top expressed genes given a user defined threshold, parameter type="expression" the selection of the X top variable genes given a user defined threshold, parameter type="variance" + gene variance is calculated using edgeR Tag-wise dispersion. The method estimates the gene-wise dispersion implementing a conditional maximum likelihood procedure. For more information please refer to edgeR Bioconductor package manual.” 3) Relating to the previous major comment 10: The legend of Figure 2 in the main manuscript has not been corrected for spelling and phrasing mistakes yet. Answer 3: The legend of Figure 2 was revised as suggested by the reviewer.
Additional Information:	
Question	**Response**
Are you submitting this manuscript to a special series or article collection?	No
Experimental design and statistics Full details of the experimental design and	Yes

statistical methods used should be given in the Methods section, as detailed in our Minimum Standards Reporting Checklist. Information essential to interpreting the data presented should be made available in the figure legends. Have you included all the information requested in your manuscript?	
Resources A description of all resources used, including antibodies, cell lines, animals and software tools, with enough information to allow them to be uniquely identified, should be included in the Methods section. Authors are strongly encouraged to cite Research Resource Identifiers (RRIDs) for antibodies, model organisms and tools, where possible. Have you included the information requested as detailed in our Minimum Standards Reporting Checklist?	Yes
Availability of data and materials All datasets and code on which the conclusions of the paper rely must be either included in your submission or deposited in publicly available repositories (where available and ethically appropriate), referencing such data using a unique identifier in the references and in the “Availability of Data and Materials” section of your manuscript. Have you have met the above requirement as detailed in our Minimum Standards Reporting Checklist?	Yes

rCASC: reproducible Classification Analysis of Single Cell sequencing data

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Abstract

Background

Single-cell RNA sequencing is an essential tool to investigate cellular heterogeneity, and to highlight cell sub-population specific signatures. Single-cell sequencing applications are now spreading from the most conventional RNAseq to epigenomics, e.g. ATAC-seq. Single-cell sequencing led to the development of a large variety of algorithms and associated tools. However, to the best of our knowledge, there are few computational workflows providing analysis flexibility and achieving at the same time functional (i.e. information about the data and the utilized tools are saved in terms of meta-data) and computational reproducibility (i.e. real image of the computational environment used to generate the data is stored) through a user-friendly environment.

Findings

rCASC is a modular workflow providing integrated analysis environment (from counts generation to cell subpopulation identification) exploiting docker containerization to achieve both functional and computational reproducibility in data analysis. Hence, rCASC provides preprocessing tools to remove low quality cells and/or specific bias, e.g. cell cycle. Subpopulations discovery can be instead achieved using different clustering techniques based on different distance metrics. Quality of clusters is then estimated through a new metric namely Cell Stability Score (CSS), which describes the stability of a cell in a cluster as consequence of a perturbation induced by removing a random set of cells from the overall cells' population. Our experiments highlight that CSS provides better cluster-robustness information than silhouette metric. Moreover, rCASC provides tools for the identification of clusters-specific gene-signature.

Conclusions

rCASC is a modular workflow with valuable new features that could help researchers in defining cells subpopulations and in detecting subpopulation specific markers. It exploits docker framework to make easier its installation and to achieve a computation reproducible analysis. Moreover, a Java Graphical User Interface (GUI), is provided in rCASC to make friendly the use of the tool even for users without computational skills in R.

Keywords

Single-cell data preprocessing, workflow, GUI, clustering, cluster stability metrics, cluster-specific gene signature.

Findings

rCASC: a single cell analysis workflow designed to provide data reproducibility.

Since the end of the 90's omics high-throughput technologies have generated an enormous amount of data, reaching today an exponential growth phase. The analysis of omics big data is a revolutionary means of understanding the molecular basis of disease regulation and susceptibility, and this resource is made accessible to the biological/medical community via bioinformatics frameworks. However, due to the increasing complexity and the fast evolution of omics methods, the reproducibility crisis [1] is becoming a very important issue [2] and there is a mandatory need to guarantee robust and reliable results to the research community [3].

Single cell analysis is instrumental to understand the functional differences existing among cells within a tissue. Individual cells of the same phenotype are commonly viewed as identical functional units of a tissue or an organ. However, single cells sequencing results [4] suggest the presence of a complex organization of heterogeneous cell states producing together system-level functionalities. A mandatory element of single cell RNAseq is the availability of dedicated bioinformatics workflows.

To the best of our knowledge, rCASC is the only computational framework, which provides both computational and functional reproducibility for an integrated analysis of single cell data, from counts generation to cell subpopulation identification. It is one of the tools developed under the umbrella of the Reproducible Bioinformatics project[1] (http://reproducible-bioinformatics.org/), an open-source community aimed to provide to biologists and medical scientists an easy-to-use and flexible framework, which also guarantees the ability to reproduce results independently by the underlying hardware, using docker containerization (computational reproducibility). Indeed, it was developed following the best practice rules for reproducible computational research, proposed in 2013 by Sandve [5]. It is also listed within the tools developed by the Italian Elixir node (https://bio.tools/rCASC).

In details all the computational tools in rCASC are embedded in docker images stored in a public repository on docker hub. Parameters are delivered to docker containers via a set of R functions, part of rCASC R github package [8]. To simplify the use of rCASC package to users without scripting experience, R functions can be controlled by a dedicated GUI, integrated in the 4SeqGUI tool

[1] The reproducible bioinformatics project was founded and it is maintained by the research team of Elixir node at University of Turin. The reproducible bioinformatics project was published on BMC Bioinformatics [6]. An example of stand-alone hardware/software infrastructure for bulk RNAseq, developed within the Reproducible Bioinformatics project, was described in Beccuti [7].

previously published by us [7], which is also available as github package [9]. rCASC is specifically designed to provide an integrated analysis environment for cell subpopulation discovery. The workflow allows the direct analysis of fastq files, generated with 10X Genomics and inDrop platforms, or count matrices. Therefore, rCASC provides raw data preprocessing, subpopulation discovery via different clustering approaches and cluster-specific genes-signatures detection. The key elements of rCASC workflow are shown in Figure 1, and the main functionalities are summarized in Methods section. A detailed description of the rCASC functions is also available in the vignettes section of rCASC github [8].

The overall characteristics of rCASC were compared with other four workflows for single-cells analysis (Figure 2): i) simpleSingleCell, Bioconductor workflow package [10]; ii) Granatum, web-based scRNA-Seq analysis suite [11]; iii) SCell, graphical workflow for single-cell analysis [12]; iv) R toolkit Seurat [13]. The comparison was based on the following elements: a) supported single-cell platforms, b) types of tools provided by the workflow, c) type of reproducibility granted by the workflow, d) tools flexibility.

rCASC is the only workflow providing support at fastq level because all the other packages require as input the processed counts table. Cell quality control and outliers' identification is available in all the workflows but Granatum. Association of ENSEMBL gene IDs to gene symbols is only provided by rCASC. All the workflows provide genes filtering tools but simpleSingleCell. All packages provide normalization procedures to be applied to raw counts data. However, rCASC is the only tool providing both Seurat specific normalization [13] and count-depth specific normalization [14]. The workflows implement different data reduction and clustering methods. rCASC integrates four clustering tools, i.e. Seurat [13] SIMLR [15], griph [16], and scanpy [17] which differ in the metrics driving the clustering analysis. Cluster stability is an important topic in Clustering (for a review see [19]). Stability measurement, taking advantage of bootstrapping, was also addressed by Hennig [20]. Specifically, Hennig uses Jaccard index to evaluate the overall stability of each cluster. In rCASC, we have implemented a cell stability score (CSS), which uses the Jaccard index to estimate the stability of each cell in each cluster. CSS provides an enhanced description of each cluster, since it allows the identification of subset of cells, in any cluster, which are particularly sensitive to perturbation of the overall dataset structure, i.e. cell bootstrapping. Moreover, the cluster stability measurement proposed by Henning was included in rCASC. Specifically, we have implemented the "clusterboot" function from the fpc R package [21], which allows the evaluation of the cluster stability using a personalized clustering function (see Supplementary file Section 5.3). To the best of our knowledge, rCASC is the only workflow performing clustering in presence of data perturbation,

i.e. removal of a subset of cells, and measuring cluster quality using Cell Stability Score (CSS is a cluster quality metrics developed by us, which measures the persistence of each cell in a cluster upon data perturbation, see Supplementary file section 5.1) and Silhouette score (SS is a cluster quality metrics measuring the consistency within clusters of data). In our experiments, CSS provides a better estimation of the cluster stability compared to that of SS (Figure 2). Gene feature selection approaches are implemented in different way in the five workflows. Granatum is the only one providing biological inference. Granatum and Seurat implements various statistical methods to detect cluster specific genes signatures (Figure 3). rCASC embeds an ANOVA-like statistics derived from EdgeR Bioconductor package [22] and Seurat/SIMLR genes prioritization procedures (see Supplementary file section 7). Visualization of genes-signatures by heatmap, coloring cells on the basis of gene expression is only provided by rCASC (see Supplementary file Figure 51). Considering reproducibility, only rCASC provides both computational and functional reproducibility. Finally, rCASC is the only one providing both a command line interface and graphical user interface (Figure 4).

Finally, rCASC was used to re-analyze the single-cell dataset from Pace paper [23]. In this paper, authors highlighted that Suv39h1-defective $CD8^+$ T-cells show sustained survival and increased long-term memory reprogramming capacity. Our re-analysis extends the information described in Pace paper, suggesting the presence of an enriched Suv39h1-defective memory subset. A complete description of the above analysis is available at section 8 of supplementary file.

Methods

Counts table generation

inDrop single-cell sequencing approach was originally published by Klein [24]. Then, the authors published the detailed protocol in Nature Methods in 2017 [25]. In rCASC, the generation of the count table starting from fastq files refers to the version 2 of the inDrop chemistry described in [25], which is commercially distributed by 1CellBio. The procedure described in the inDrop github [26] is embedded in a docker image. rCASC function *indropIndex* allows the generation of the transcripts index required to convert fastq in counts, and *indropCounts* function converts reads in UMI counts. 10XGenomics Cellranger is packed in a docker image and the function *cellrangerCount* converts fastq to UMI matrix using any of the genome indexes with *cellrangerIndexing* function. Detailed description about the counts table generation is available in Supplementary file section 2.

Counts table exploration and manipulation

rCASC provides various data inspection and preprocessing tools.

genesUmi function generates a plot where the number of detected genes are plotted for each cell with respect to the number of UMI (Figure 5A,C).

mitoRiboUmi calculates the percentage of mitochondrial/ribosomal genes with respect to the total number of detected genes in each cell and plots percentage of mitochondrial genes with respect to percentage of ribosomal genes. Furthermore, cells are colored on the basis of the number of detected genes (Figure 5B, D). *mitoRiboUmi* allows to identify cells with low information content, i.e. those cells with a little number of detectable genes, e.g. < 100 genes/cell, little ribosomal content and high content of mitochondrial genes, which indicate cell stress [27].

The function *scannobyGtf* uses ENSEMBL gtf and the R package refGenome to associate gene symbol with the ENSEMBL gene ID. Furthermore, *scannobyGtf* allows one to remove mitochondrial/ribosomal genes (Figure 5A, C) and "stressed" cells detectable with *mitoRiboUmi* function (Figure 5B, D).

The function *lorenzFilter* embeds the Lorenz statistics developed by Diaz [12], a cell quality statistics correlated with cell live-dead staining (see Supplementary file sections 3.3). Specifically, the outlier filtering for single-cell RNA-seq experiments designed by Diaz estimates which genes are expressed at background levels in each sample, then samples with significantly high background levels are discarded [12].

As counts table preprocessing steps, we implemented the functions *checkCountDepth/scnorm* to detect the presence of sample specific count–depth relationship [14] (i.e. the relationship existing between transcript-specific expression and sequencing depth) and to adjust the counts table for it. Specifically, *checkCountDepth* initially executes a quantile regression, thus estimating the dependence of transcript expression on sequencing depth for every gene. Then, genes with similar dependence are aggregated (see Supplementary file section Figure 21). *Scnorm*, after executing *checkCountDepth*, performs a new quantile regression to estimate scale factors within each group of genes. Then, sequencing depth adjustment is done within each group using the estimated scale factors. Furthermore, we added two other functions *recatPrediction* and *ccRemove*, which are based respectively on the paper of Liu [28] and Barron [29]. The function *recatPrediction* organizes the single cell data to reconstruct cell cycle pseudo time-series and it is used to understand if a cell cycle effect is present. The above function embeds reCAT software [28], which models the reconstruction of time-series as a traveling salesman problem, thus identifying the shortest possible cycle by passing through each cell exactly once and returning to the start. Since the traveling salesman problem is a NP-hard problem, reCAT is based on a heuristic algorithm, which is used to find the solution.

ccRemove function is instead based on the work of Barron and Li [29] and embeds their scLVM (single-cell latent variable model) algorithm, which uses a sophisticated Bayesian latent variable

model to reconstruct hidden factors in the expression profile of the cell-cycle genes. This algorithm is able to remove cell-cycle effect from real scRNA-Seq datasets. Thus, *ccRemove* is used to mitigate the cell cycle effect of the inter-samples transcriptome, when it is detected by *recatPrediction* function (see Supplementary file sections 3.6 ad 3.8).

Clustering

For the identification of cell subpopulations we implemented four approaches: Seurat [13], SIMLR [15], griph [16] and scanpy [17]. Seurat is a toolbox for single-cell RNAseq data analysis. We implemented in rCASC one of the clustering procedures present in Seurat toolbox. The function *seuratPCAEval* has to be run before executing the clustering program to identify the 'metafeatures', i.e. the subset of PCA components describing the relevant source of cells' heterogeneity, to be used for clustering. *seuratBootstrap* function implements data reduction and clustering. Specifically, cells undergo to global scaling normalization, i.e. LogNormalize method, and scaling factor 10000. Subsequently, a linear dimensional reduction is done using the range of principal components defined with *seuratPCAEval*. Then, clustering is performed using the cell PCA scores. The Seurat clustering procedure, embedded in *seuratBootstrap*, is based on the Louvain modularity optimization algorithm. Differently SIMLR implements a k-mean clustering, where the number of clusters (i.e. k) is taken as input. SIMLR, requires as input raw counts $\log_{10}$ transformed. SIMLR is capable of learning an appropriate cell-to-cell similarity metric from the input single-cell data and to exploit it for the clustering task. In the learning phase SIMLR identifies a distance metric that better fits the structure of the data by combining multiple Gaussian kernels [15]. Thus, the tool can deal with the large noise and drop-out effects of single-cell data, which could not easily fit with specific statistical assumptions made by standard dimension reduction algorithms [15]. The function *simlrBootstrap* controls the clustering procedure and the function *nClusterEvaluationSIMLR*, a wrapper for the R package griph (Graph Inference of Population Heterogeneity) [16], is exploited to estimate the (sub)optimal number "k" of clusters. Griph clustering [16] is based on Louvain modularity. Griph algorithm is closer to agglomerative clustering methods, since every node is initially assigned to its own community and communities are subsequently built by iterative merging. Also scanpy [17] uses for clustering a heuristic method based on modularity optimization.

We developed, for Seurat, SIMLR, griph and scanpy, a procedure to measure the cluster quality on the basis of data structure. The rationale of our approach is that cells belonging to a specific cluster should be little affected by changes in the numerosity of the dataset, e.g. removal of 10% of the total number of cells used for clustering. Thus, we developed a metrics called CSS (Cell Stability Score), which describes the persistence of a cell in a specific cluster upon Jackknife resampling and therefore offers a peculiar way of describing cluster stability. Detailed description of CSS metrics is available

in Supplementary file at section 5.1. CSS is embedded in *seuratBootstrap, simlrBootstrap, scanpyBootstrap* and *griphBootstrap*.

Feature selection

To select the most important features of each cluster we implemented in the *anovaLike* function the edgeR ANOVA-like method for single cells [22] and in the functions *seuratPrior* and *genesPrioritization/genesSelection* respectively the Seurat and SIMLR genes prioritization methods. *hfc* function allows the visualization of the genes prioritized with the above methods as heatmap and provides plots of prioritized genes in each single cell (Figure 6).

Scalability

To estimate the scalability of rCASC clustering we used the GSE106264 dataset made of 10,035 cells and published by Pace and coworkers in 2018 [23] and the 10,000/33,000/68,000 cells PBMC human datasets, available at 10xGenomics repository (www.10xgenomics.com). We randomly generated from the 10035 cells (27998 ENSEMBL GENE IDs) the following subsets of cells: 400, 600, 800, 1000, 2000, 5000. Moreover for the subsets with more than 600 cells we randomly sampled the genes: 10000, 8000, 6000, 4000, 2000, 1000, 800. We run SIMLR, tSne, griph and Seurat using 160 permutations within SeqBox hardware [7]: Intel i7 3.5GHz (4 cores), 32 GB RAM and 500 GB SSD disk. SIMLR resulted to be the slowest and, given the above hardware implementation, it cannot allocate for the analysis more than 2000 cells (Figure 7A left panel). All the other tools were able to handle up to 5000 cells within the limit of 32 GB of RAM available in the hardware setting used in this analysis. Computation time was nearly linear for all tools till 1000 cells. Only griph clustering resulted to be nearly insensitive to the increasing number of cells (Figure 7A). We extended, for Seurat, griph and scanpy, the scalability analysis to 10K, 33K, 68K and 101K cells, using 10,000/33,000/68,000 cells from PBMC human datasets, available at 10xGenomics repository (www.10xgenomics.com), and 101,000 cells dataset, made assembling the above mentioned 33,000 and 68,000 PBMC datasets. The analysis was executed on a SGI server (10 x CPU E5-4650 2.4GHz (16 cores), 1TB RAM, 30 TB SATA raid disk) allocating 40 threads for each analysis. Scanpy outperforms the other two methods and griph behaves slightly better than Seurat (Figure 7A right panel).

The computing time as function of increasing number of genes has a quite limited effect on the overall computing time (Figure 7B).”

Availability and requirements

Project name: rCASC: reproducible Classification Analysis of Single Cell sequencing data

Project home page: https://github.com/kendomaniac/rCASC; https://github.com/mbeccuti/4SeqGUI

Operating system: Linux

Programming language: R and JAVA

Other Requirements: None

License: The GNU Lesser General Public License, version 3.0 (LGPL-3.0)

Any restrictions to use by non-academics: None

Authors' contributions

LA and FC equally participated to write R scripts, to create the majority of docker images, to package the workflow and release code. MB wrote the Java and C++ code, and acted as corresponding author. NL implemented scanpy and extended the Java GUI. MA and MO prepared the single-cell data to be used as examples of the workflow functionality. GR prepared the dockers for fastq to counts table conversion. SR revised all packages and generated the docker files for docker images maintenance and further development. GDL gave scientific advices and provided an unpublished dataset for MAIT resting and activated T-cells (generated with Fluidigm C1 platform) to investigate genes detection limits in 3'end sequencing technologies and whole transcript sequencing. RAC and LP equally oversaw the project and gave scientific advices. All authors read, contributed and approved the final manuscript.

Supplementary material

rCASC_supplementary_file.pdf

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Figure legend

Figure 1: rCASC workflow. Blue boxes indicate preprocessing tools. Yellow boxes define clustering tools. Green box indicates genes-signatures tools.

Figure 2: Cell Stability Score versus Silhouette Score calculated on Pace's dataset (see Supplementary file section 8) using SIMLR over a set of number of clusters ranging between 5 and 8. A) Cell Stability Score violin plot. Mean value and data dispersion suggest that the best number of clusters is 5. Cells remain in the same cluster about 80% of the times, repeating the clustering upon random removal of 10% of the cells. B) Silhouette Score violin plot. Mean value of the SS distribution does not provide clear evidences that one clustering condition is better that another. Furthermore, the dispersion of the SS value shrinks as the number of the clusters increases.

Figure 3: Comparison between the analysis features available in rCASC and in the other single-cell analysis workflows.

Figure 4: rCASC graphical interface within 4seqGUI. A) Counts table generation menu: this set of functions is devoted to the conversion of fastq to a counts table. B) Counts table manipulation menu: this set of functions provides inspection, filtering and normalization of the counts table. C) Clustering menu: these functions allow the use of SIMLR, tSne, Seurat, griph and scanpy to group cells in subpopulations. D) Feature selection menu: this set of functions allows the identification of cluster-specific subsets of genes and their visualization using heatmaps.

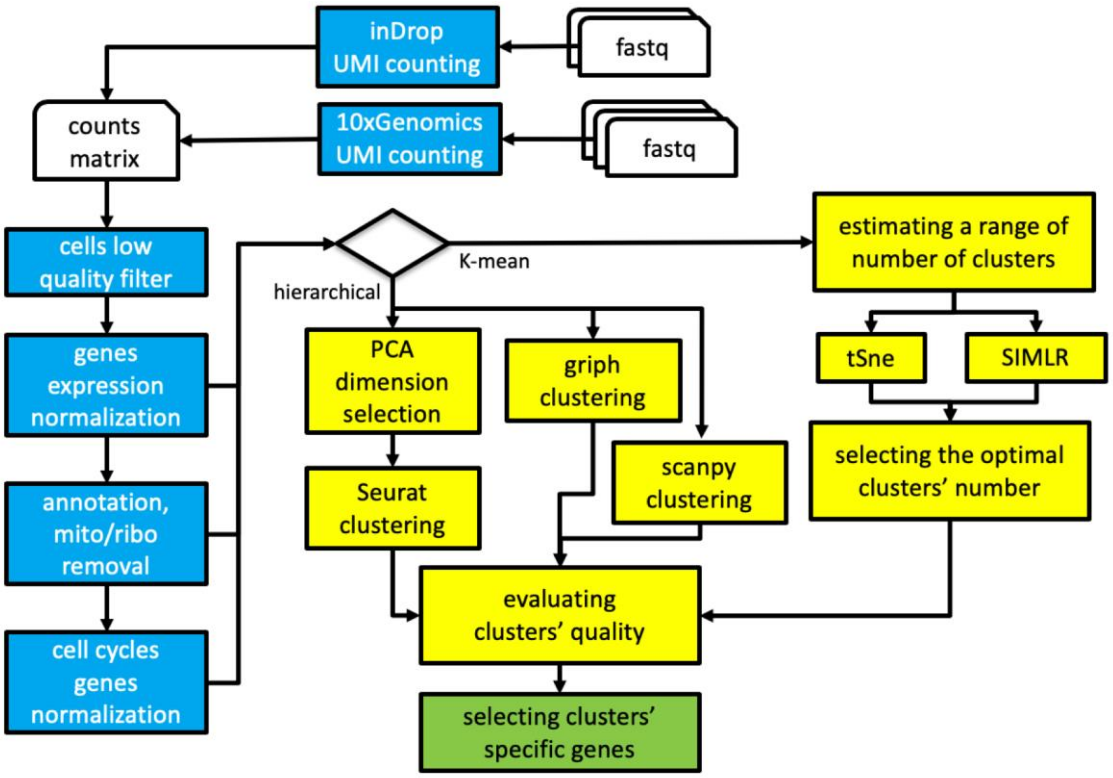
Figure 5: *genesUmi* plots the number of detectable genes in each cell (a cell is called present if it is supported by at least N UMI/reads, suggested values are N=3 for UMI or N=5 for smart-seq sequencing [30]) with respect to the number of UMI/cell. *mitoRiboUmi* calculates the percentage of mitochondrial and ribosomal genes with respect to the total number of detected genes in each cell. It plots % of mitochondrial genes with respect to % of ribosomal genes. Furthermore, cells are colored on the basis of the number of detected genes: A) *genesUmi* plot for resting CD8+ T-cells [23], sequencing average 83,000 reads/cell. B) *mitoRiboUmi* plot for resting CD8+ T-cells [23]. The majority of the cells with less than 100 detected genes groups together and they are characterized by high relative percentage of mitochondrial genes and low relative percentage of ribosomal genes. Remaining cells are characterized by few detectable genes, 100÷250 genes/cell, with a percentage of ribosomal genes greater than 30%. C) *genesUmi* plot for Listeria activated CD8+ T-cells [23],

sequencing average 83,000 reads/cell, it is notable the activated cells show a wider range of detectable genes with respect to resting cells (B). D) *mitoRiboUmi* plot for Listeria activated CD8+ T-cells [23]. The majority of the cells are characterized by more the 100 genes and they show low percentage of mitochondrial genes and percentage of ribosomal genes between 15% and 35%. The remaining cells, with less than 100 detected genes groups together and are characterized by high relative percentage of mitochondrial genes and low relative percentage of ribosomal genes.

Figure 6: Heatmap and cell expression plot for prioritized genes. A) Heatmap for the set of 577 genes selected for Pace datasets (see Supplementary file section 8) by SIMLR prioritization. B) Nkg7 CPM expression in the cell clusters. Nkg7 is expressed in activated T-cells (clusters 1, 2, 4, 5) [31] but not in resting T-cells (cluster 3).

Figure 7: Scalability analysis of the clustering tools implemented in rCASC. A) Time required to perform 160 permutations as function of increasing number of cells on approximately 20,000 genes. Left panel: SIMLR, tSne, Seurat and griph clustering up to 5,000 cells was executed on a SeqBox [7] (1 x CPU i7-6770HQ 3.5 GHz (8 cores), 32 GB RAM, 1TB SSD). Right panel: Seurat, griph and scanpy analyses were extended until 101,000 cells using an SGI server (10 x CPU E5-4650 2.4GHz (16 cores), 1TB RAM, 30 TB SATA raid disk). B) Time required to perform 160 permutations as function of increasing number of genes on a set of 800 cells, analysis performed on a SeqBox.

Figure 1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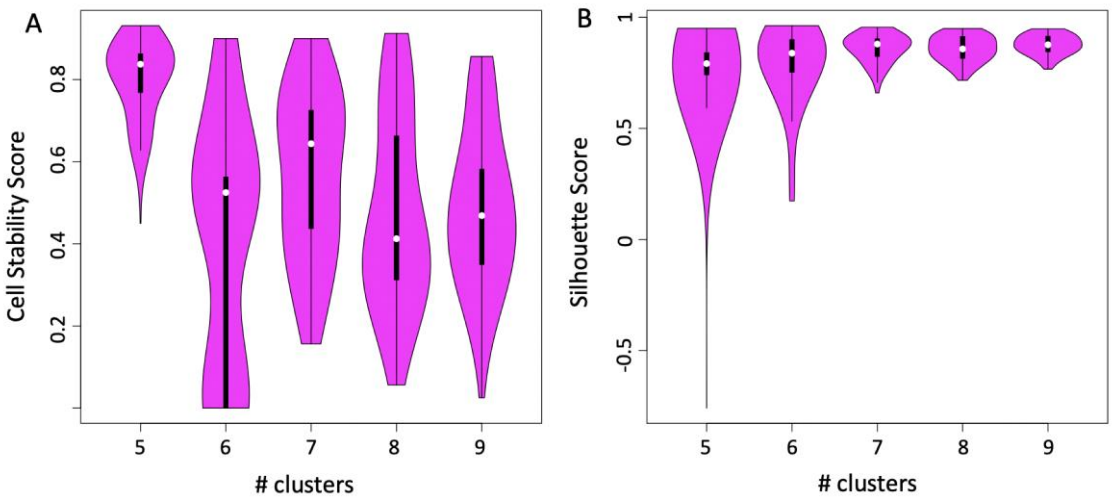
genes expression normalization
annotation, mito/ribo removal
cell cycles genes normalization
hierarchical
K-mean
estimating a range of number of clusters
PCA dimension selection
griph clustering
tSne
SIMLR
Seurat clustering
scanpy clustering
selecting the optimal clusters' number
evaluating clusters' quality
selecting clusters' specific genes

Figure 2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Silhouette Score
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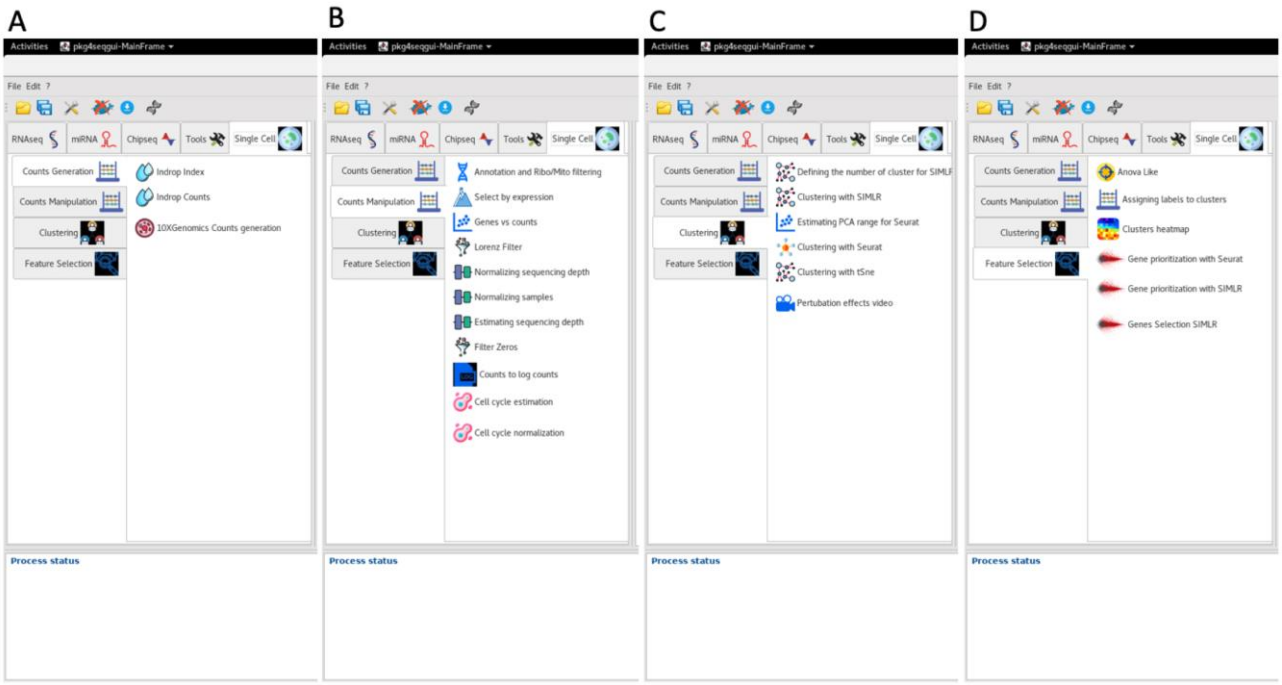
clusters

Figure 3

		rCASC (stand alone)	simpleSingleCell (stand alone)	Granatum (web)	Scell (stand alone)	Seurat (stand alone)
Platforms		10Xgenomics, inDrop, counts table	counts table	counts table	counts table	counts table
Tools	*Fastq conversion in counts table*	Y	-	-	-	-
	Quality Control / Outlier Filtering	Y	-	Y	Y	Y
	Annotation	ENSEMBL ID -> Gene Symbol	-	-	-	-
	Genes filter	Y	-	Y	Y	Y
	Data normalization	Y	Y	Y	Y	Y
	Cell cycle bias removal	Y	-	-	Y	Y
	Data dimensionality reduction	Y	Y	Y	Y	Y
	Supported clustering methods	tSne, SIMLR, Seurat PCA	Walktrap	Non-negative matrix factorization, K-mean (Euclidean), K-mean (tSne)	PCA, k-means, Gaussian mixture, Minkowski weighted k-means, DBSCAN	PCA, tSne, ica, dmap
	Cluster quality score	Silhuette, Cell Stability Score	-	-	-	-
	Features selection and visualization	Y	Y	Y	-	-
	Supported methods	ANOVA-like (edgeR), SIMLR Seurat genes prioritization	filtering on expression	NODES, SCDE, EdgeR, Limma	-	wilcox, bimod, roc, t-test, tobit, negbinom, MAST, DESeq2
	Biological inference	-	-	Y	-	-
Reproducibility	*Functional reproducibility*	Y	Y	-	-	Y
	Computational reproducibility	Y	-	Y	Y	-
Flexibility	*line command execution*	Y	Y	-	-	Y
	graphical interface	Y	-	Y	Y	-

Figure 4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Clustering
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Annotation and Ribo/Mito filtering
Select by expression
Genes vs counts
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Normalizing sequencing depth
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Counts to log counts
Cell cycle estimation
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Defining the number of cluster for SIMLR
Clustering with SIMLR
Estimating PCA range for Seurat
Clustering with Seurat
Clustering with tSne
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Assigning labels to clusters
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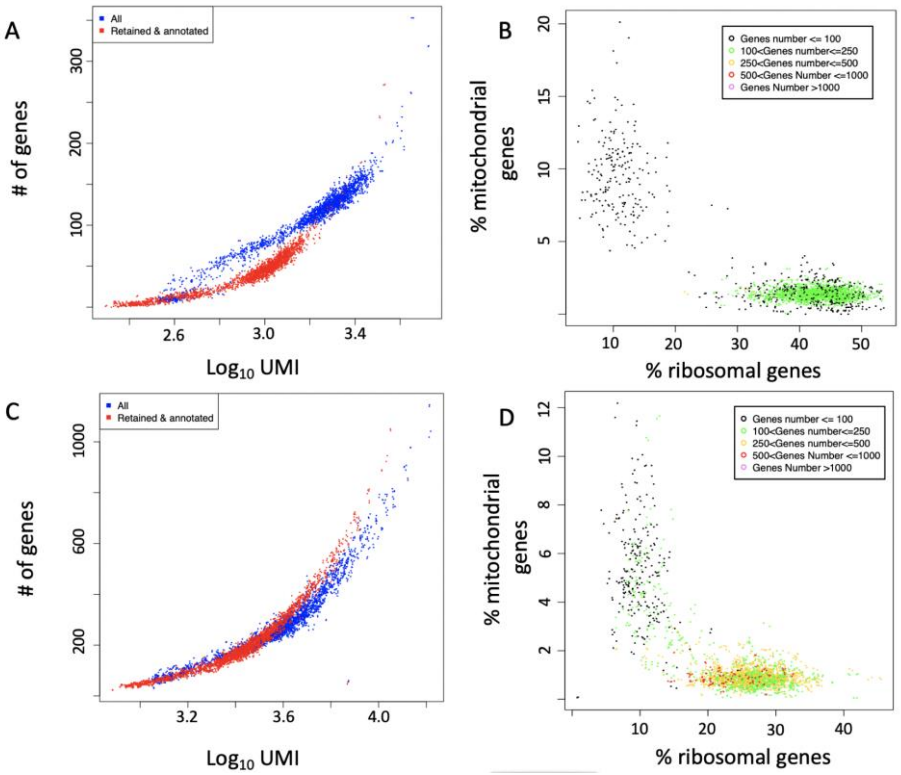
Gene prioritization with Seurat
Gene prioritization with SIMLR
Genes Selection SIMLR
Process status

Figure 5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Genes Number >1000
% mitochondrial genes
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Figure 6

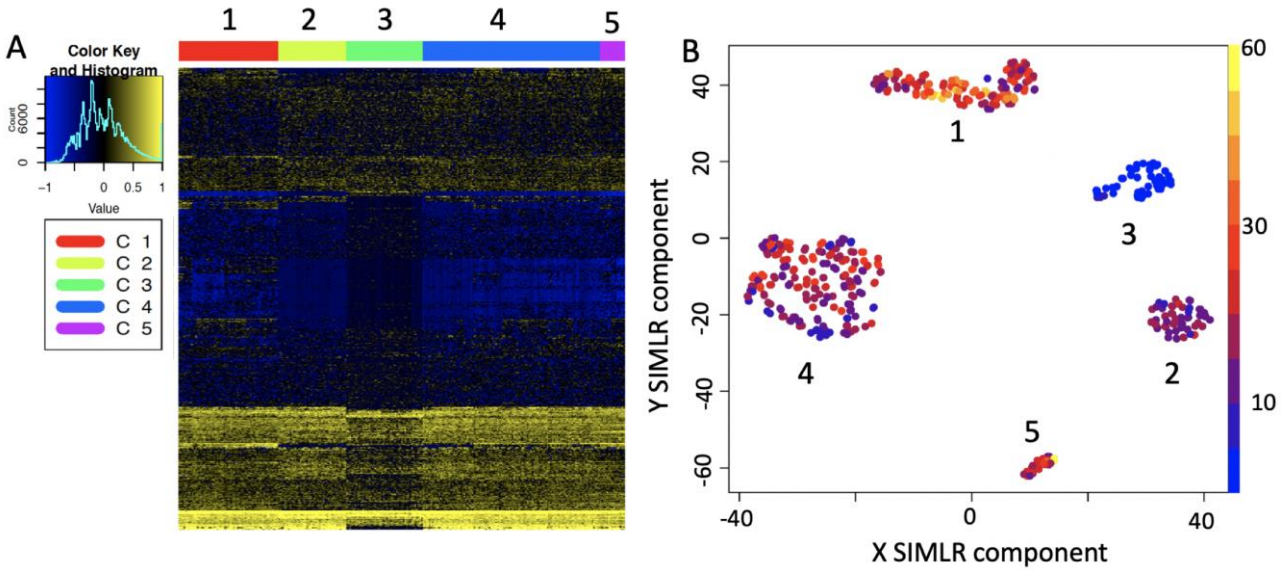
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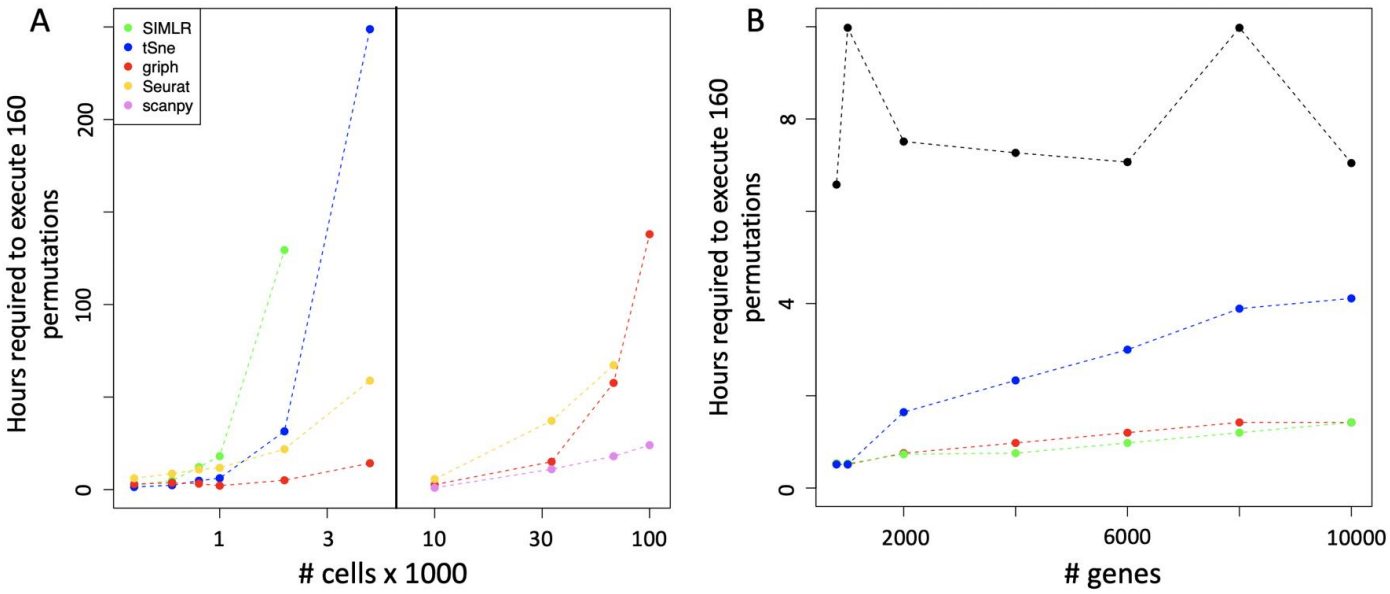
Figure 7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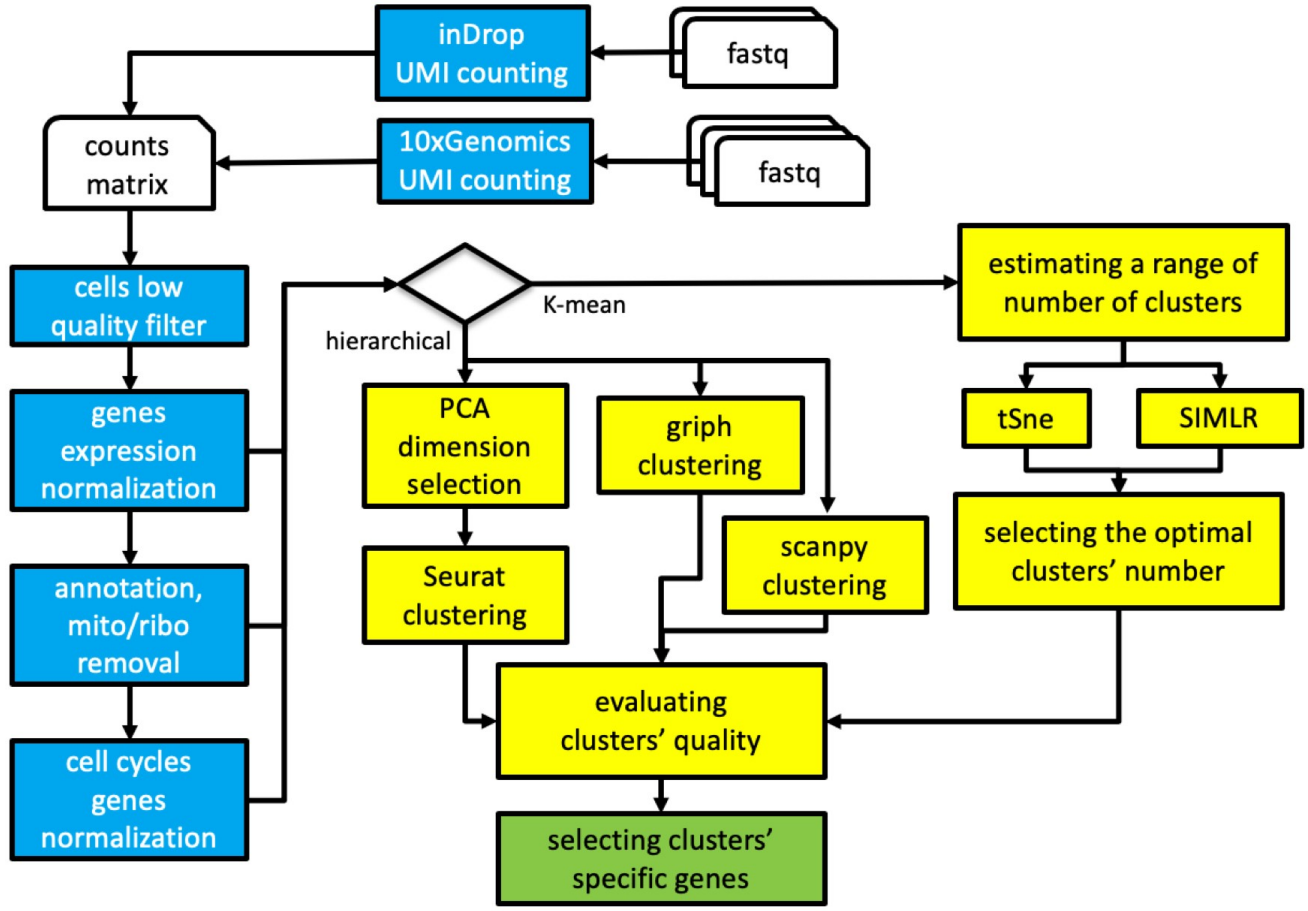
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genes
normalization
evaluating
clusters' quality
selecting clusters'
specific genes

Fig.1

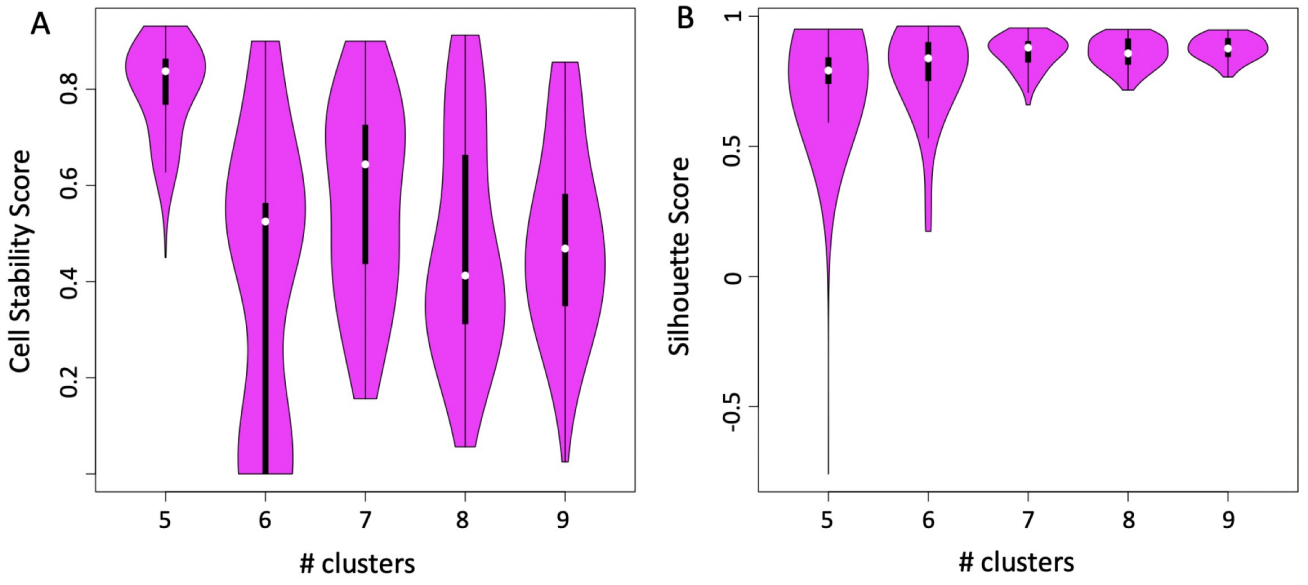
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Fig.2

		rCASC (stand alone)	simpleSingleCell (stand alone)	Granatum (web)	Scell (stand alone)	Seurat (stand alone)
Platforms		10Xgenomics, inDrop, counts table	counts table	counts table	counts table	counts table
Tools	*Fastq conversion in counts table*	Y	-	-	-	-
	Quality Control / Outlier Filtering	Y	-	Y	Y	Y
	Annotation	ENSEMBL ID -> Gene Symbol	-	-	-	-
	Genes filter	Y	-	Y	Y	Y
	Data normalization	Y	Y	Y	Y	Y
	Cell cycle bias removal	Y	-	-	Y	Y
	Data dimensionality reduction	Y	Y	Y	Y	Y
	Supported clustering methods	tSne, SIMLR, Seurat PCA	Walktrap	Non-negative matrix factorization, K-mean (Euclidean), K-mean (tSne)	PCA, k-means, Gaussian mixture, Minkowski weighted k-means, DBSCAN	PCA, tSne, ica, dmap
	Cluster quality score	Silhuette, Cell Stability Score	-	-	-	-
	Features selection and visualization	Y	Y	Y	-	-
	Supported methods	ANOVA-like (edgeR), SIMLR Seurat genes prioritization	filtering on expression	NODES, SCDE, EdgeR, Limma	-	wilcox, bimod, roc, t-test, tobit, negbinom, MAST, DESeq2
	Biological inference	-	-	Y	-	-
Reproducibility	*Functional reproducibility*	Y	Y	-	-	Y
	Computational reproducibility	Y	-	Y	Y	-
Flexibility	*line command execution*	Y	Y	-	-	Y
	graphical interface	Y	-	Y	Y	-

Fig.3

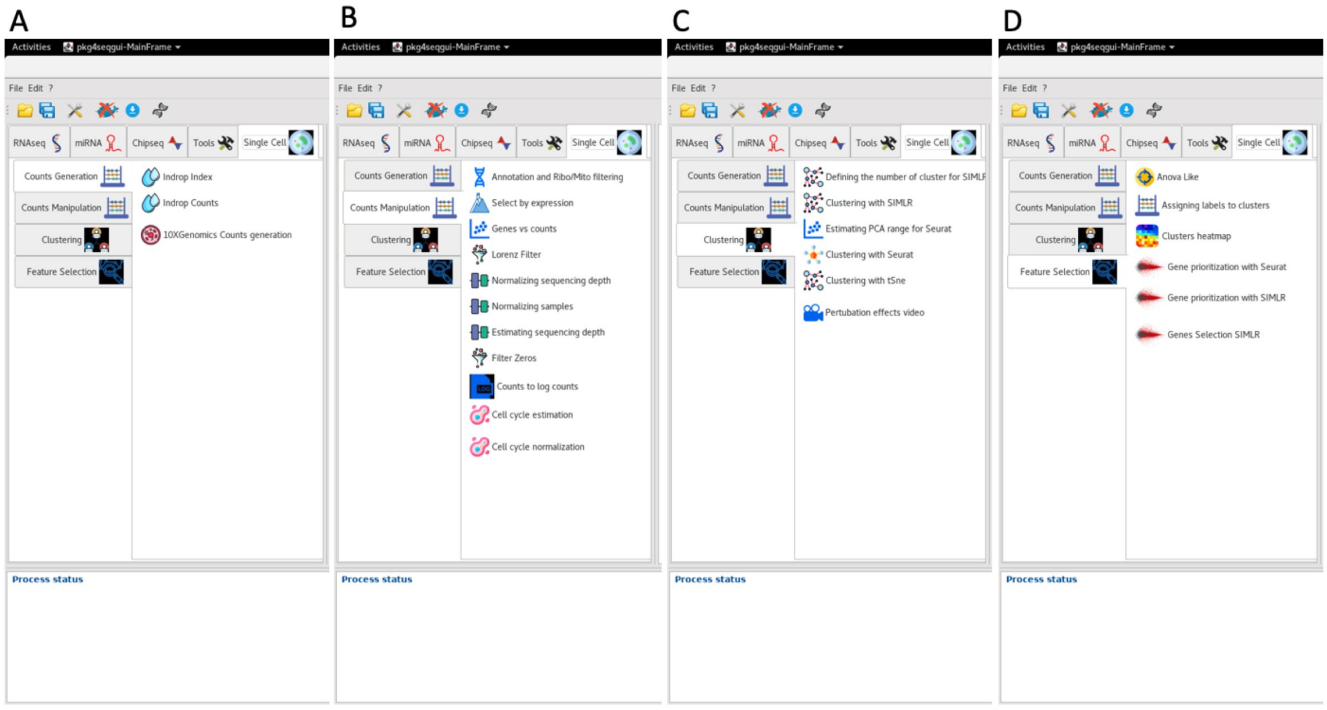

Fig.4

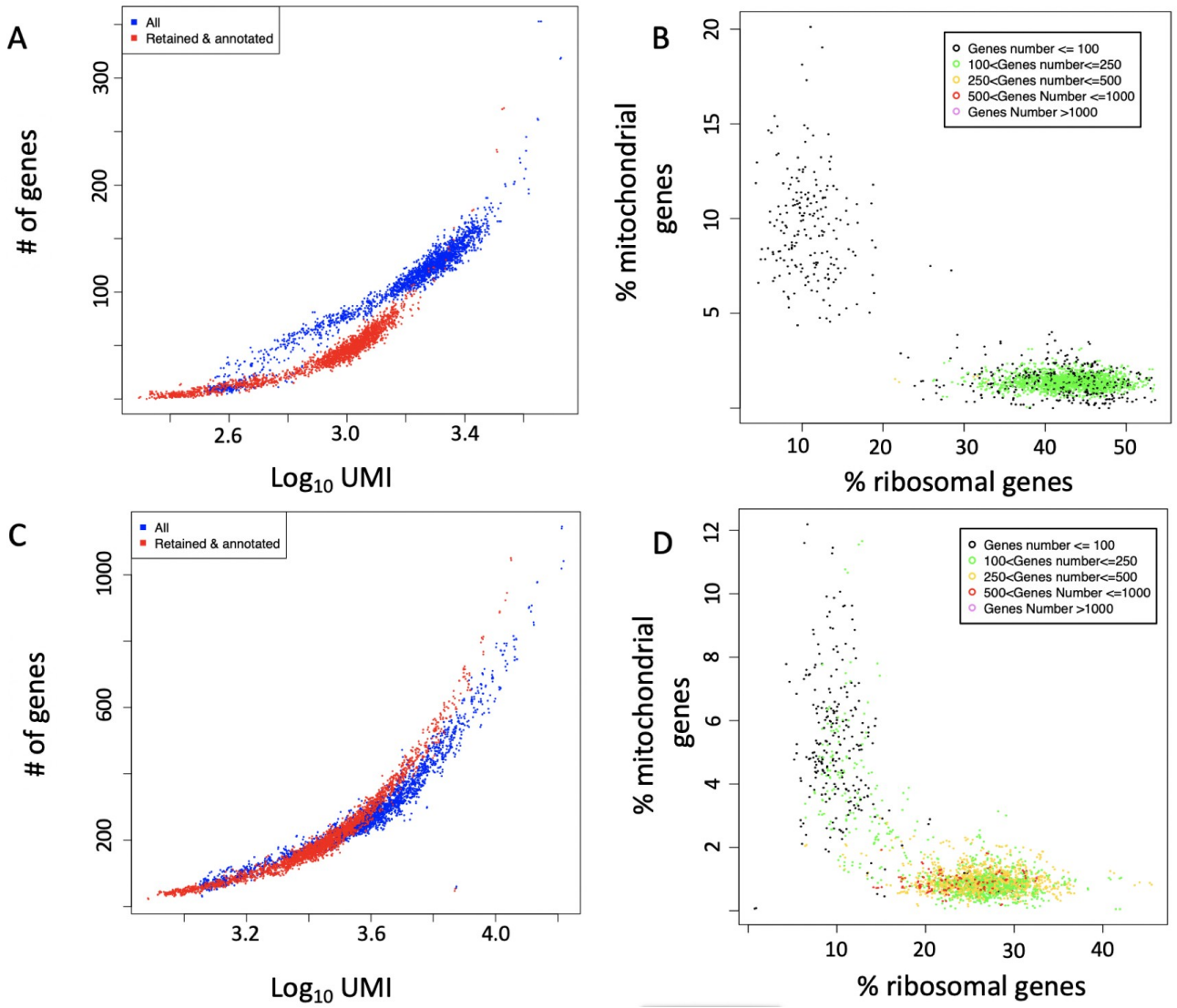
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Fig.5

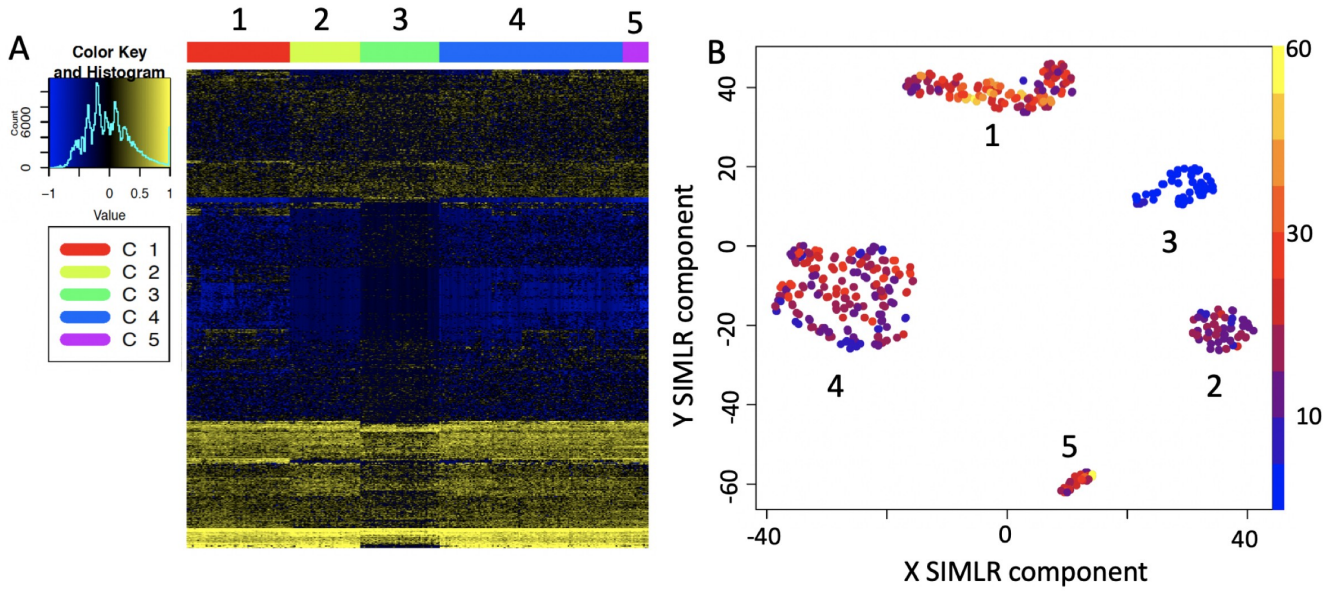
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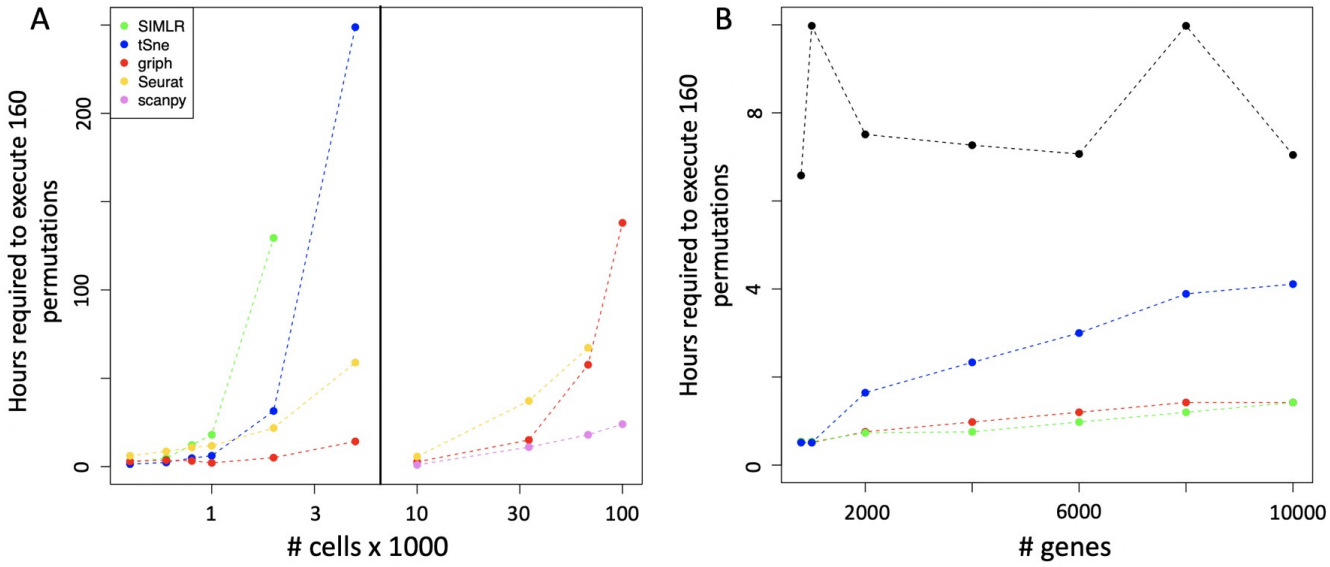
Fig.6


A
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Hours required to execute 160 permutations
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Fig.7

Rebuttal letter for the paper:

rCASC: reproducible Classification Analysis of Single Cell sequencing data.

Luca Alessandrì, Francesca Cordero, Marco Beccuti, Maddalena Arigoni, Martina Olivero, Greta Romano, Sergio Rabellino,Nicola Licheri, Gennaro De Libero, Luigia Pace and Raffaele A Calogero

Dear Editor,
First of all, we wish to thank the reviewers for their valuable comments and useful suggestions which helped us to substantially improve the paper and its associated tool.

Hereafter we report our answers to the reviews' comments.

Reviewer #1

First, I would like to thank the authors for the hard and great work they did to address the different major aspect previously mentioned. Notably, they evaluated the time complexity of random, artificially created datasets, using 160 permutations, using 6 different sizes for both features (800 to 10000) and number of cells (from 400 to 5000). They also rewritten several sections of the manuscript such as the CSS part, and corrected different concerning aspects (such as the "supervised clustering" designation). They also simplified their pipeline and added a "mini" option to be able to download only a few Docker containers on all the available containers of rCASC which are enough to provide basic functionalities to handle single-cells. Finally, they investigated the relationship between stability and cluster significant with additional experiments. In the other hand, I am a little bit disappointed by the performance of rCASC in term of scaling-up. Using rCASC, it seems difficult to process and cluster more than 5K cells with a reasonable machine, such as a personal computer of a cluster node (with RAM up to 64GiG), and in a reasonable time (less than 24-48h). Unfortunately, I was expecting this result because rCASC uses a lot of tools that at some point require the computation of a step having a polynomial complexity. For example, SIMLR require the computation of a cell-cell distance matrix. Increasing the number of cells gives rapidly a matrix that either takes a very long time to compute /and/or cannot be loaded in the RAM of the computer. Being able to process a larger number of cells is I think a very important aspect of any new single-cell bioinformatic pipeline because A) the technology is growing rapidly and we can expect a larger amount of cells to be processed in the near future, and B) more and more single cell datasets are available leading to meta-analyses combining multiple single-cell datasets.
My first comment is: is there any settings of rCASC that can be used to process a dataset larger than 5K cells? Ideally 100K (See for example Scanpy pipeline that can process up to 1M) would be a good number. However, I think that at least 10K cells is a minimum. For example, it seems that Griph is a method the less greedy in term of resources used. Then, maybe a specific set of settings designated for "very_large_dataset" processing can be used with Griph to be able to compute larger dataset? In that case, not all the options of rCASC need to be used (especially the ones using a lot of resources). Is it possible to adapt the stability metric to such datasets? However, if the authors think that clustering datasets larger than 5K cells is out of scope of this study what would be the reason to justify the non-scalability of the method? I agree that rCASC allows a very rigorous and complete analysis of a smaller dataset but is the processing of 1 to 5K cells enough considering the single-cell RNA-Seq field evolution (it is an open question)?

Answer #1:
We incorporated in rCASC the clustering tools *scanpy* and *griph* as required by the reviewer. Moreover, we tested the performance of rCASC using *Seurat, griph,* and *scanpy* tools considering 10K, 35K, 68K and 101K cells. The analysis was executed on an SGI server (10 x CPU E5-4650 2.4GHz (16 cores), 1TB RAM, 30 TB SATA raid disk) allocating 40 threads for each analysis.
The results of this comparison highlight that *griph* works slightly faster than *Seurat* tool. However, both these tools do not scale well when more than 68K cells are analyzed. Differently *scanpy* shows the best performance up to 101K cells. Hence, figure 7 was redraw to report the new results, and its caption "*Scalability analysis of clustering tools implemented in rCASC. A) Time required to perform 160 permutations as function of increasing number of cells on a set of 27998 genes. B) Time required to perform 160 permutations as function of increasing number of genes on a set of 800 cells.*" was updated in the following way:
"Scalability analysis of the clustering tools implemented in rCASC. A) Time required to perform 160 permutations as function of increasing number of cells on approximately 20,000 genes. Left panel: SIMLR, tSne, Seurat and griph clustering up to 5,000 cells was executed on a SeqBox [7] (1 x CPU i7-6770HQ 3.5 GHz (8 cores), 32 GB RAM, 1TB SSD). Right panel: Seurat, griph and scanpy analyses were extended until 101,000 cells using an SGI server (10 x CPU E5-4650 2.4GHz (16 cores), 1TB RAM, 30 TB SATA raid disk). B) Time required to perform 160 permutations as function of increasing number of genes on a set of 800 cells, analysis performed on a SeqBox."

Moreover, Scalability section in the main manuscript "To estimate the scalability of rCASC clustering we used the GSE106264 dataset made of 10035 cells and published by Pace and coworkers in 2018 [21]. We randomly sampled the 10035 cells (27998 ENSEMBL GENE IDs) to obtain the following subsets of cells: 400, 600, 800, 1000, 2000, 5000.

Starting from the 800 cells set we randomly sampled the genes: 10000, 8000, 6000, 4000, 2000, 1000, 800. We run SIMLR, tSne, griph and Seurat using 160 permutation within SeqBox hardware [7]: Intel i7 3.5GHz (4 cores), 32 GB RAM and 500 GB SSD disk. SIMLR resulted to be the slowest and, given the above hardware implementation, it cannot allocate for the analysis more than 2000 cells (Figure 7A). All the other tools were able to handle up to 5000 cells within the limit of 32 GB of RAM available in the hardware setting used in this analysis. Computation time was nearly linear for all tools till 1000 cells. Only griph clustering resulted to be nearly insensitive to the increasing number of cells (Figure 7A). The computing time as function of increasing number of genes has a quite limited effect on the overall computing time (Figure 7B)." was updated as follows:
"To estimate the scalability of rCASC clustering we used the GSE106264 dataset made of 10,035 cells and published by Pace and coworkers in 2018 [23] and the 10,000/33,000/68,000 cells PBMC human datasets, available at 10xGenomics repository (www.10xgenomics.com).
We randomly generated from the 10035 cells (27998 ENSEMBL GENE IDs) the following subsets of cells: 400, 600, 800, 1000, 2000, 5000. Moreover, for the subsets with more than 600 cells we randomly sampled the genes: 10000, 8000, 6000, 4000, 2000, 1000, 800. We run SIMLR, tSne, griph and Seurat using 160 permutations within SeqBox hardware [7]: Intel i7 3.5GHz (4 cores), 32 GB RAM and 500 GB SSD disk. SIMLR resulted to be the slowest and, given the above hardware implementation, it cannot allocate for the analysis more than 2000 cells (Figure 7A left panel). All the other tools were able to handle up to 5000 cells within the limit of 32 GB of RAM available in the hardware setting used in this analysis. Computation time was nearly linear for all tools till 1000 cells. Only griph clustering resulted to be nearly insensitive to the increasing number of cells (Figure 7A). We extended, for Seurat, griph and scanpy, the scalability analysis to 10K, 33K, 68K and 101K cells, using 10,000/33,000/68,000 cells from PBMC human datasets, available at 10xGenomics repository (www.10xgenomics.com), and 101,000 cells dataset, made assembling the above mentioned 33,000 and 68,000 PBMC datasets. The analysis was executed on a SGI server (10 x CPU E5-4650 2.4GHz (16 cores), 1TB RAM, 30 TB SATA raid disk) allocating 40 threads for each analysis. Scanpy outperforms the other two methods and griph behaves slightly better than Seurat (Figure 7A right panel).
The computing time as function of increasing number of genes has a quite limited effect on the overall computing time (Figure 7B)."

Finally, the supplementary file was updated adding all the new functionalities implemented as consequence of the reviewer's requests.

Reviewer #2

The authors have now extensively revised the manuscript and added a number of useful functions to the existing rCASC package. By doing so, the authors addressed the majority of my previous comments. However, there remain a few minor comments that should be addressed prior to publication.

1) Relating to the previous major comment 1: The publication associated to the griph package can now be cited as: Serra et al., Self-organization and symmetry breaking in intestinal organoid development. Nature (2019).

Answer 1:
we updated the reference.

2) Relating to the previous major comment 7: It would be useful to provide more details on which variability measure (variance, squared coefficient of variation or a regression residual) is used to rank genes based on their variability in the topx function while setting type="variance".

Answer 2:
we updated the description of topx function in the supplementary material specifying that user can decide to use the top expressed/variable genes for clustering. The function **topx** provides the selection of the X top expressed genes given a user defined threshold.

Hence, in the supplementary material the following sentences were revised:

"For clustering purposes user might decide to use the top expressed/variable genes. The function **topx** provides two options:
the selection of the X top expressed genes given a user defined threshold, parameter type="expression"
the selection of the X top variable genes given a user defined threshold, parameter type="variance"

+ gene variance is calculated using edgeR Tag-wise dispersion. The method estimates the gene-wise dispersion implementing a conditional maximum likelihood procedure. For more information please refer to edgeR Bioconductor package manual."

3) Relating to the previous major comment 10: The legend of Figure 2 in the main manuscript has not been corrected for spelling and phrasing mistakes yet.

Answer 3: The legend of Figure 2 was revised as suggested by the reviewer.