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METHOD ARTICLE

REVISED Cell motility influences microfluidics capturing in scRNA-seq

[version 2; peer review: 1 approved, 2 approved with reservations]

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Abstract

Microfluidic isolation methods for single-cell RNA sequencing (scRNA-seq) have primarily been designed for immotile cells, with limited consideration for motile cells, such as those with flagella or cilia. By studying the encapsulation efficiency of the flagellated *Trypanosoma brucei* using the 10x Genomics platform, we found that maintaining parasites at room temperature results in a low encapsulation yield. We implemented a rapid cooling method to 0°C prior to encapsulation, which reduced parasite motility and prevented undesired transcriptomic changes. This allowed for a representative scRNA-seq dataset, avoiding the disproportionate loss of the most motile forms. This study highlights the challenges of using motile cells in microfluidic systems and the biases caused by losing specific subpopulations, emphasizing the need for optimized protocols for non-standard mammalian cells.

Plain language summary

Single-cell RNA sequencing (scRNA-seq) is a powerful technique used to study individual cells, but most methods have been designed for non-motile cells. This can be a challenge when working with motile cells, like *Trypanosoma brucei*, a flagellated parasite responsible for sleeping sickness. In our study, we found that these parasites are poorly captured in microfluidic systems, such as the 10x Genomics platform, when kept at room temperature. Their movement reduces the efficiency of encapsulation, leading to biased results. To overcome this, we developed a simple cooling method that quickly lowers the temperature to 0°C before processing. This reduces parasite motility, improving capture rates while minimizing unwanted changes in gene expression. By applying this approach, we obtained a representative transcriptomic dataset, ensuring that all parasite forms were included. Our findings highlight the importance of adapting microfluidic techniques for motile cells to avoid losing key subpopulations and

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ensure accurate biological insights.

Keywords
scRNA-seq, Trypanosoma brucei, parasite, microfluidics, encapsulation, motility, flagellum



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REVISED **Amendments from Version 1**

In this revised version, we have addressed all reviewer comments and improved the clarity, transparency, and robustness of the manuscript.

No changes were made to the title or abstract. However, two new authors were added due to their valuable discussions and contributions in addressing the reviewers' comments.

We deposited an additional processed dataset ("InVivo_RT_QUENCHED.rds") in Zenodo to improve transparency and reproducibility. The supplementary material was also updated to include legends for all supplementary videos. In addition, we corrected the mention of a non-existent supplementary video in the text.

The main text was revised to clarify several aspects:

- Results: We expanded the discussion regarding the expected proportions of slender versus stumpy forms at day 6 post-infection and clarified differences in classification criteria relative to previous literature. We improved the clarity of the GO term analysis by explicitly aligning the direction of comparisons with the differential expression analysis. In addition, we included a targeted analysis of temperature-shock-related genes. Table 1 now includes standard deviations, and Figure 3 was updated to improve visualization. We also removed a redundant paragraph at the end of one Results section following a reviewer comment.
- Discussion: We clarified the rationale for applying identical QC thresholds across samples and expanded the Discussion to acknowledge that factors beyond motility (e.g., ambient RNA or overall cell quality) may contribute to the observed differences.
- Methods: We clarified that sequencing saturation was reached in all samples.

These revisions strengthen the interpretation of the results while maintaining the original conclusions.

Any further responses from the reviewers can be found at the end of the article

Introduction

In the pursuit of unravelling cellular diversity, single-cell RNA sequencing (scRNA-seq) has emerged as a transformative tool. scRNA-seq is defined as an advanced technology, used to measure the gene expression of individual cells. It comprises several steps, such as cell isolation, library preparation, sequencing and data analysis. Studying single cell transcriptomes has enabled researchers to not only study gene expression in thousands of individual cells simultaneously[1] but also to understand heterogeneity within diverse cell populations, such as antibiotic-associated cellular states under antibiotic perturbation in bacteria[2] or a unique immune response against malaria parasite.[3]

A variety of methods have been developed for scRNA-seq.[4] The central difference between them lies in how individual cells are isolated and barcoded. Microfluidics and droplet-based technologies eliminated the need for sorting and fixing cells, reducing cell stress during preparation, minimizing potential changes to gene expression and also greatly improving cell isolation efficiency.[5] In 10x Genomics (one of the most used technologies) microfluidics are used to encapsulate individual cells within nanoliter-sized droplets, along with Gel Beads and an oil emulsion, which are called GEMs. These Gel Beads carry two different barcodes: one called 10X, unique for each cell, and the other called Unique Molecular Identifier (UMI), unique for each transcript, allowing a more accurate quantification of transcript levels and enabling the elimination of artifacts such as PCR duplicates.[6]

Some cell characteristics pose challenges for encapsulation and barcoding. For example, yeast cells have a rigid cell wall that needs to be lysed with special enzymes,[7] and cells larger than 50 μm cannot pass through some microfluidic channels.[8] Since scRNA-seq is relatively new (first paper was published in 2009[9]) the sample preparation has been reported already as a source of artifacts, and leading to false discoveries, related with stress-related genes expression.[10] One possible solution for this problem has been to keep the cells as inactive as possible, which is achieved by keeping them in cold before encapsulation[11] or cryopreserve them,[12] as typically done in bulkRNA-seq. Another obstacle could be cell motility; however, this issue that has not been widely reported, as cell loss is typically linked to washes or other technical problems,[13] making it difficult to demonstrate.

There are several motile cells in nature. Highly motile sperm cells are found across diverse animal groups, including reptiles, birds, and mammals. Some immune cells also exhibit motility, though at much lower rates - such as naïve T cells at 6.2 μm/min, or CD8 T cells at around 4.3 to 5.2 μm/min.[14] On the other hand, among parasites, Leishmania[15] and Trypanosoma,[16] for example, are highly motile. *Trypanosoma brucei,* a highly motile flagellated parasite,[17] crosses barriers in different hosts, including blood vessels in mammals[18] and the proventriculus (or cardia) in the tsetse fly.[19]

A highly motile behaviour that is also observed in culture. The flagellum is essential for motility and plays a pivotal role in multiple facets of development, transmission, and pathogenesis.[20,21] The displacement of the flagellum also influences the parasite's morphology and consequent speed of displacement.[16,20] In the mammalian host, quiescent stumpy forms have a shorter flagellum and slower velocity (10–20 μm/s) compared to proliferative slender forms, with a longer flagellum and higher velocity (20–50 μm/s).[22,23] Additionally, parasites capable of reaching the brain and crossing the blood–cerebrospinal fluid barrier[18] exhibit longer free flagella and increased velocity (50–100 μm/s).[23,24] Similarly, in the tsetse fly, life cycle stages and flagellum length influence swimming velocity.[23]

In this study, we investigated how cell motility affects single-cell isolation and barcoding in a microfluidic system (10x Genomics). By examining two different stages of *Trypanosoma brucei* parasites with varying motilities, we observed that highly motile slender forms were underrepresented in the final dataset compared to the less motile stumpy forms. We found that immobilizing the parasites prior to encapsulation by rapidly cooling the samples enhanced cell retention of motile parasites by up to 70% compared to when parasites were stored at room temperature. This approach results in a representative scRNA-seq data and underscores the importance of optimizing protocols for non-standard cells in microfluidic devices.

Methods

Trypanosoma brucei cell lines

Mice were infected by *Trypanosoma brucei* EATRO1125 strain, AnTat1.1E clone, chimeric triple reporter cell-line[25] expressing the red-shifted firefly luciferase protein PpyREH9, tagged with TdTomato and TY1. For *in vitro* scRNA-seq analysis EATRO1125 AnTat1.1 GFP::PAD1utr was used, in which a GFP gene is followed by a PAD1 3′ UTR that confers maximum expression in parasites that have initiated differentiation to stumpy forms.

Parasite isolation from blood

After 5 or 6 days of infection (depending on the time point of the experiment), mice were sacrificed with CO_2. Blood was removed by heart puncture (1 mL per mouse) and deposited in a 10 mL falcon with 2 mL of 2% CGA (Citrate Glucose Anticoagulant, S4641-25G and G8270-100G Sigma- Alrich) and centrifuged for 10 min at 2000 rpm. The layer between the red blood cells and serum was removed by pipetting and mixed with 3 mL of the Separation Buffer from the DEAE sepharose™ Fast Flow column (Merk GE17-0709-01). After the selection with the DEAE column, parasites were counted using a disposable haemocytometer and diluted at the desired concentration in the buffer 1X PBS (Thermo Fisher Scientific, 14190250) supplemented with 1% D-glucose (PSG) (Sigma-Aldrich, G8270-100G) and 0.04% bovine serum albumin (BSA) (Sigma-Alrich A9418-50G) at room temperature. For microscopy imaging, parasites were resuspended at 1–10 million parasites in 1 mL and for the scRNA-seq experiment 1×10^5 parasites were resuspended in 1 mL.

For the RT scRNA-seq protocol, parasites were left in a 1.5 mL microtube with 1% D-glucose (PSG) and 0.04% bovine serum albumin (BSA) at room temperature until the moment of single cell isolation in the Chromium Controller (RT tubes). For the QUENCHED samples, 500 μL of the original tube at RT were transferred into another 1.5 mL microtube and immediately placed in a bath of 100% EtOH with dry ice for 5–7 seconds, until the thermometer marked 0 degrees. Next, this sample was transferred to a water bath with ice and kept there until the moment of single cell isolation.

Single-cell RNA sequencing protocol

For each scRNA-seq sample, a total of 7000 cells from the mixed sample were loaded into the Chromium Controller (10x Genomics) to capture individual cells with unique barcoded beads. Libraries were prepared using the Chromium Single Cell 3′ GEM, Library & Gel Bead Kit v3.1 Dual index (10x Genomics). Total cDNA was measured with the LabChip GX Touch Nucleic Acid Analyzer and the DNA kit (Part #: 760569). Library preparation was performed following the protocol from 10x Genomics. Sequencing was performed with Novogene with the Illumina NovaSeq™ 500 platform to a total of at last 100Gb per sample. At least 20000 reads per cell was required for each sample, reaching at least 80% saturation.

Single-cell RNA sequencing quality control

Illumina reads were aligned to the reference genome[26] combined with the genes encoding for luciferase protein PpyREH9, TdTomato, TY1 tag,[25] GFP, the VSG AnTat1.1E (closest sequence GenBank: X01843.1) and a list of early expressed VSG ORF.[27] Reads were mapped onto the genome using cellranger-7.2.0 count function with the default settings. Low-quality cells were removed by filtering for low unique genes detected (<800), high proportion of kDNA (>2%) and high proportion of rRNA (>8%).[26]

Single-cell RNA sequencing normalization and clustering

The data was normalized following the procedure outlined in Briggs *et al.* 2021.[26] A low resolution (0.2) was selected for identification of the two most distinct populations. As the samples were sequenced together, no integration was performed to enable a comprehensive analysis of the differences between them. Replicates from the QUENCHED condition indicated the absence of batch effects in the data, with observed changes only attributable to biological factors.

Stumpy and slender score

The list of genes from slender and stumpy form were generated from Briggs *et al.* 2021[26] data. This set of genes was utilized to calculate the average expression stumpy or slender scores using the AddModuleScore function from Seurat as indicated in Larcombe *et al*. 2023.[28]

Pseudobulk analysis

All counts were aggregated to the sample level using "AggregateExpression" from Seurat, across four different samples: slender and stumpy from QUENCHED condition, and slender and stumpy from the RT condition.

Microscope videos

To record parasite motility on video, 10 µl of parasites were placed on a slide. For the QUENCHED parasites, slides were pre-chilled on ice for at least 10 min before acquiring the videos rapidly, ensuring that the parasites remained cool and unaffected by laser temperature. To record parasite motility in GEMs, the training kit from 10x Genomics was utilized, and parasites were collected as described in the Parasite Isolation section of the bloodstream form.

All the videos presented in this article were taken in a 3i Marianas SDC (spinning disc confocal) microscope (Intelligent Imaging Innovations, equipped with a Yokogawa CSU-X1 confocal scanner and a Photometrics Evolve 512 EMCCD camera)[29] (https://imm.medicina.ulisboa.pt/facility/bioimaging/doku.php?id=3i_marianas_sdc). Transmitted light and laser line 561 nm were used to image the parasites and its TdTomato expression. The objectives used in these acquisitions were a 10x EC Plan-Neofluar (0.3 NA; 5.50 mm WD), and a 20x Plan-Apochromat (0.8 NA; 0.55 mm WD). A hundred images were obtained in each time lapse, with an acquisition rate of 2 frames per second. For all acquisitions, the software used was 3i Slidebook reader v.6.0.22 (https://www.intelligent-imaging.com/slidebook) allowing export of images in TIFF format. TIFF documents were then processed using ImageJ 1.52a Java 1.8.0_112 [64-bit] (https://imagej.net/ij/), that also can be used to acquire the videos.

All experiments were conducted at the Rodent Platform of the Gulbenkian Institute for Molecular Medicine.

Results

Parasites at room temperature are less efficiently encapsulated

To assess how *T. brucei* motility affects encapsulation in GEMs and vice versa, parasites were isolated from mice on day five post-infection (mostly slender forms[30]) and kept at room temperature (RT) until encapsulation. Encapsulated parasites were observed under an optical microscope, and we noticed that the physical process of encapsulation did not compromise the morphology of the parasites and individual motility (Supplementary Video 1). However, some parasites showed a long displacement and high velocity, suggesting they may have not been properly encapsulated or they erupted from the GEMs (Supplementary Video 2).

To increase the probability of parasites being properly encapsulated, we tested whether rapidly lowering the temperature would decrease parasite motility. For that, a parasite suspension was rapidly cooled by submersion in a bath of 100% EtOH with dry ice for a few seconds, reaching 0 degrees Celsius, followed by immediate placement on ice, not letting the parasites freeze. This quenching is intended to rapidly reduce the metabolic activity and therefore the motility, while preventing cold-induced stress. This method is very commonly used in metabolomics to avoid changes in the metabolome of the cells.[31] As a control, an equal amount of parasite suspension was left at room temperature (RT). As expected, we found that QUENCHED parasites showed a severe impairment in motility, with the parasites appearing almost immotile (Supplementary Video 3) in contrast to parasites at RT, which showed a typical beating of the flagellum (Supplementary Video 4).

To test if differences in motility would translate into differences in cell recovery, parasites were isolated from blood six days post-infection and split into two groups: QUENCHED or RT. We had previously observed that on this day of infection, the blood is populated by a mixture of stumpy and slender forms.[30] For each condition, we prepared two technical replicates. The four samples underwent encapsulation, cDNA amplification and processed for library preparation: QUENCHED 1, QUENCHED 2, RT1, RT2 (Figure 1A). Libraries were sequenced by Illumina. Sequencing reads were aligned with CellRanger. After alignment, differences in quality between QUENCHED and RT samples were

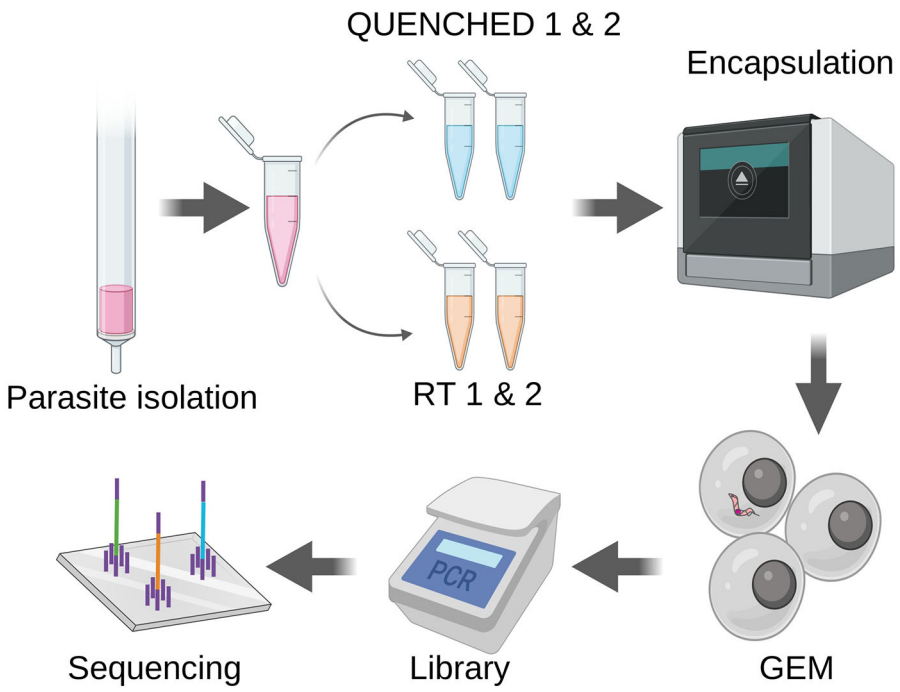

Figure 1. Motile parasites are less efficiently encapsulated. Parasites were isolated from the blood of three mice six days post-infection and purified through chromatography. Half of the parasite suspension underwent fast cooling to 0 degrees, using dry ice and ethanol (QUENCHED), while the other half remained at room temperature (RT). Both sets of samples underwent encapsulation using the Chromium controller, followed by library preparation and sequencing as recommended by 10x Genomics.

Table 1. Raw data vs filtered data.

	RAW DATA			After QC		
Sample	**Gene/cell ± SD**	**UMI/cell ± SD**	**Total cells**	**Gene/cell ± SD**	**UMI/cell ± SD**	**Total cells**
QUENCHED1	91.83 ± 168.42	123.69 ± 261.91	70380	1047.82 ± 237.75	1641 ± 429.46	1324
QUENCHED2	102.36 ± 163.46	137.26 ± 255.81	84261	1079 ± 284.47	1536.5 ± 522.78	1383
RT1	54.83 ± 161.69	91.71 ± 309.19	15396	1085.66 ± 320.64	1905 ± 655.49	145
RT2	59.24 ± 156.24	99.76 ± 252.46	84732	1074.89 ± 259.54	1721.14 ± 467.19	698

The table presents the number of genes per cell, unique molecular identifiers (UMIs) per cell, and total cell count for each sample before (RAW DATA) and after quality control (AFTER QC) with the standard variation. "QUENCHED1" and "QUENCHED2" refer to samples processed using the quenching method, while "RT1" and "RT2" were prepared using a standard room-temperature (RT) approach. QC filtering significantly reduced the total number of cells while increasing the detected genes and UMIs per cell, indicating improved data quality.

observed (Table 1). These numbers come directly from the raw matrix output from CellRanger, meaning that all barcoded cells are included. However, it is interesting that this raw data already shows differences, particularly in the number of genes per cell, which is twice as high in the QUENCHED samples than in RT samples (Table 1).

For quality control, we eliminated cells with fewer than 800 genes. This threshold was chosen based on previous studies analyzing bloodstream forms in scRNA-seq data, in which cells typically present around 1000 genes per cell.[26,28,32] We applied the same cutoff for both conditions since all cells originated from the same sample as technical replicates (Table 1).

After filtering, we found that the number of cells in QUENCHED samples was approximately threefold higher (1324 cells in QUENCHED1 and 1383 in QUENCHED2) than in RT samples (145 in RT1 and 698 in RT2) (Table 1). From the initial input of approximately 7000 cells per sample, we recovered around 20% of cells in the QUENCHED samples and less than 6% in the RT samples. Given the low number of replicates (n=2), it is expected that the difference in recovery rates is not statistically significant. Consistently, during library preparation, we noticed a higher yield of cDNA (around 50%

more) in the QUENCHED samples compared to the RT samples (Table S1). This suggests a more efficient encapsulation of QUENCHED parasites than those kept at RT, resulting in a great number of correctly barcoded parasites.

The recovery rate in 10x Genomics workflows is highly variable. While 10x advertises a recovery rate of around 60%,[33] significant heterogeneity is observed across studies, largely influenced by various technical conditions. For *Trypanosoma brucei*, other studies have reported a wide range of recovery rates, from 16% to 40%.[26,28,32] In our study, the recovery rate from the QUENCHED condition (20%) falls within this range, whereas the RT condition shows a notable drop to 6%. We conclude that samples stored at RT yielded fewer GEMs with an acceptable transcriptomic cell profile than samples quenched,[26,28,32] thereby decreasing the final yield of the scRNA-seq experiment.

Quenching improves retention of slender forms

Given stumpy forms are less motile,[22] we wondered if these forms are more efficiently encapsulated than slender forms. Given that a parasite population isolated from blood six days post infection is typically composed of a mixture of slender and stumpy forms,[30] we used the four samples from this study to compare the encapsulation yield of slender and stumpy forms in each sample.

To confirm that our four samples contained a mixture of slender and stumpy forms, we merged the four datasets and visualized them using Uniform Manifold Approximation and Projection (UMAP)[34] reduction. The four merged samples revealed the presence of two clusters (Figure 2A), which correspond to slender and stumpy forms using previously established gene signatures for these two life cycle forms (Table S2)[26] (Figure 2B). Plotting the expression of three of these marker genes (PAD2, ZC3H20 and PYK1) helped us to visualise the two life cycle stages (Figure 2C). We noticed that the actively transcribed Variant Surface Glycoprotein gene AnTat1.1E (manually added to the reference genome along with other VSG genes) was downregulated (Figure 2C, Figure S1A), as previously reported[35] in bulk RNA-seq data. Thus, this gene can also help the validation of slender/stumpy stages early in infection when VSG switchers are not detected in scRNA-seq data. We also observed differences in UMI and gene counts between the two stages, with the stumpy forms showing a higher number of detected genes and transcripts than the slender forms (Figure S1B). Although this may seem counterintuitive, the downregulation of the active VSG (Variant Surface Glycoprotein) in stumpy forms may desaturate the sequencing, allowing for the detection of a higher number of genes in stumpy forms that in slender forms.

Segregation of the UMAP based on the type of sample shows that RT samples have almost no cells in the slender cluster relative to QUENCHED samples (Figure 2D). In both replicates of the QUENCHED samples, we detected 224 and 304 stumpy forms and 1100 and 1079 slender forms (Figure 2E). Conversely, when parasites were incubated at RT, in the two replicates we detected 43 and 347 stumpy forms and only 102 and 351 slender forms (Figure 2E). This means that when parasites were kept at RT, the proportion of sequenced slender vs stumpy was 54:46%, which is markedly different from the 80:20% distribution observed in the QUENCHED samples (Figure 2F). In other words, at RT we lost on average 79% of slender forms and 26% of stumpy forms relative to fast cooling conditions (453 vs 2179 total slender forms; 390 vs 528 total stumpy forms). Given that slender forms are more motile than stumpy forms[22] and motility is reduced at lower temperatures (Supplementary Videos 3–5), quenching probably facilitated the recovery of less motile slender forms within the GEMs and thus the final sequenced population of parasites is more representative of the original expected population in the blood.

Slender forms are more sensitive to temperature changes than stumpy forms

Next, we evaluated the impact of the temperature on the transcriptome of slender and stumpy forms separately. To do so, we conducted a pseudobulk analysis of slender or stumpy form parasites in each temperature condition, followed by a correlation analysis of the transcript levels (measured as mean gene counts normalized with the total number of cells in each condition). We observed that the transcriptomes of slender forms in QUENCHED vs RT are highly correlated (correlation coefficients of 0.93). The transcriptomes of stumpy forms are also highly correlated 0.95 (Figure S2A and B). As expected, these correlations are both higher than the correlation between the transcriptomes of slender versus stumpy forms (0.91) (Figure S2C). These analyses indicate that fast cooling does not have a major impact on the transcriptome of neither slender nor stumpy forms.

However, we can detect differences between conditions, especially in the slender forms, where most RT parasites form an independent cluster (Figure 3A). To identify the genes affected by temperature in slender or stumpy forms, we performed a differential expression analysis and selected those with a log fold-change between conditions equal or higher than 0.5 and a p-value lower or equal than 0.05. In slender forms, we found 935 differentially expressed genes (from a total of 8930, i.e. ~10%) between QUENCHED and RT conditions, while in stumpy forms, 261 genes (~3%) show differential expression (Figure 3B-C and Table S3 and Table S4).

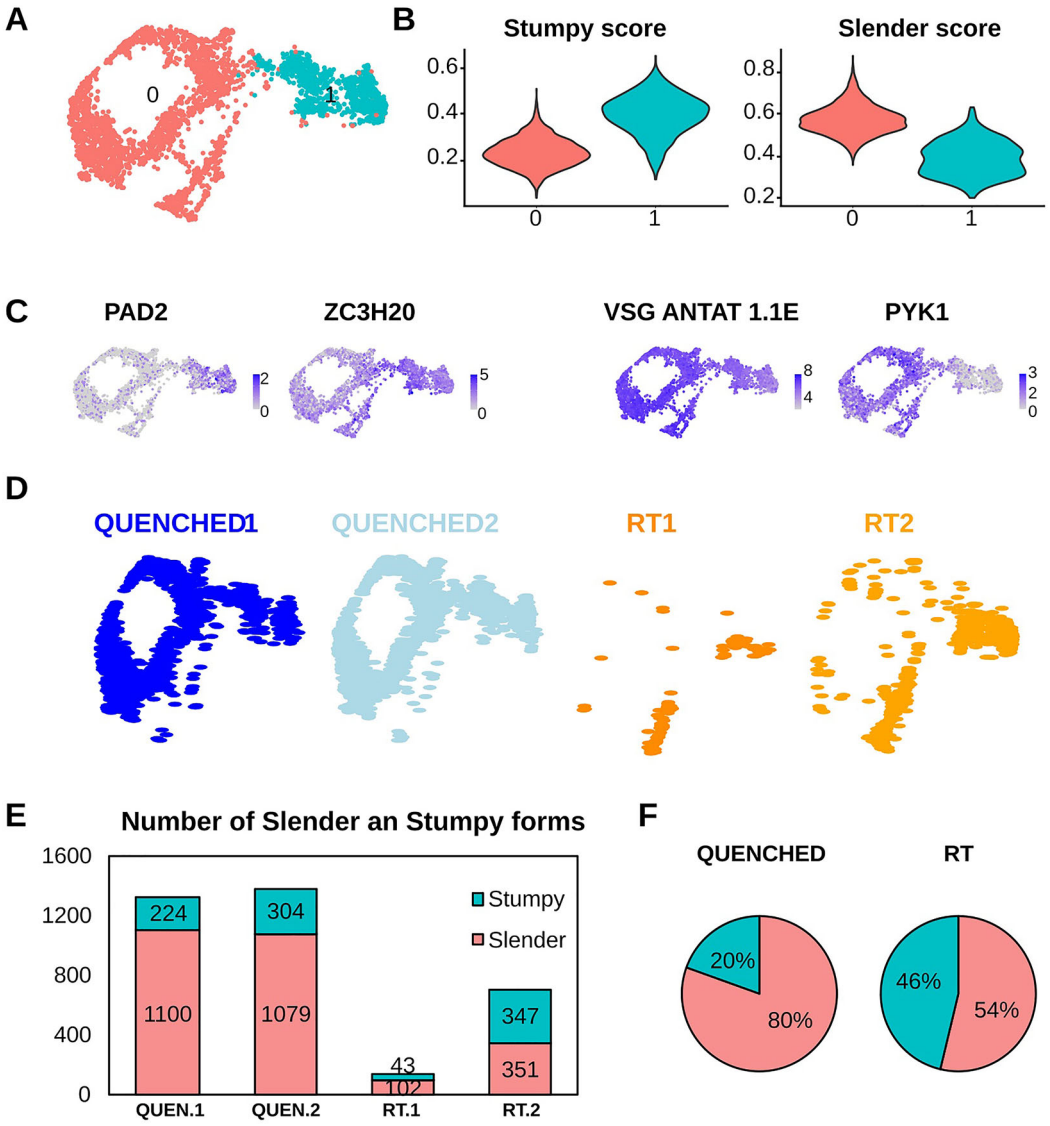

Figure 2. Quenching improves retention of slender form parasites. (A) Uniform Manifold Approximation and Projection (UMAP) plot of four merged samples showing two unsupervised clusters (cluster 0 in red and cluster 1 in cyan). (B) Violin plots from panel (A) that show the score of the cells from cluster 0 or cluster 1 as "stumpy" or "slender" with the genes showed in Tabel S1. The "slender score" and "stumpy score" consist of the average expression of associated marker genes for each cluster identified in A. (C) UMAPs coloured by transcript counts for two stumpy markers (EP1 and PAD2) and two slender markers (PYK1 and VSG AnTat1.1E). Scales show raw transcript count per cell. (D) Same UMAP plot from panel A but divided in the four idents that composes it: in blue the QUENCHED samples and in orange the RT samples. (E) Number of parasites belonging to the stumpy (cyan) or slender (red) stages in the four samples QUENCHED1, QUENCHED2, RT1, RT2 (colored as panel A). (F) Pie chart indicating the percentage of slender and stumpy forms found in the sum of two replicates at each temperature condition.

Since in slender forms, we detected more genes being upregulated in the RT condition, we decided to perform GO term analysis of these genes in RT relative to QUENCHED condition; this analysis revealed an enrichment of terms involved in DNA replication (GO:0006260), nucleocytoplasmic transport (GO:0006913), and microtubule-based movement (GO:0007018), suggesting enhanced replication, protein transport, and cytoskeletal activity in RT conditions (Figure 3D). Importantly, no GO terms associated to stress were detected (such as GO: 0009409, GO:0070417). Amongst the downregulated genes in slender forms, we did not find a GO term exclusive of this group of genes. This could be because of the low number of genes analysed. To ensure we did not miss any differentially expressed gene due to the limitations of GO term analysis, we manually analysed the predicted function of all differentially expressed genes in

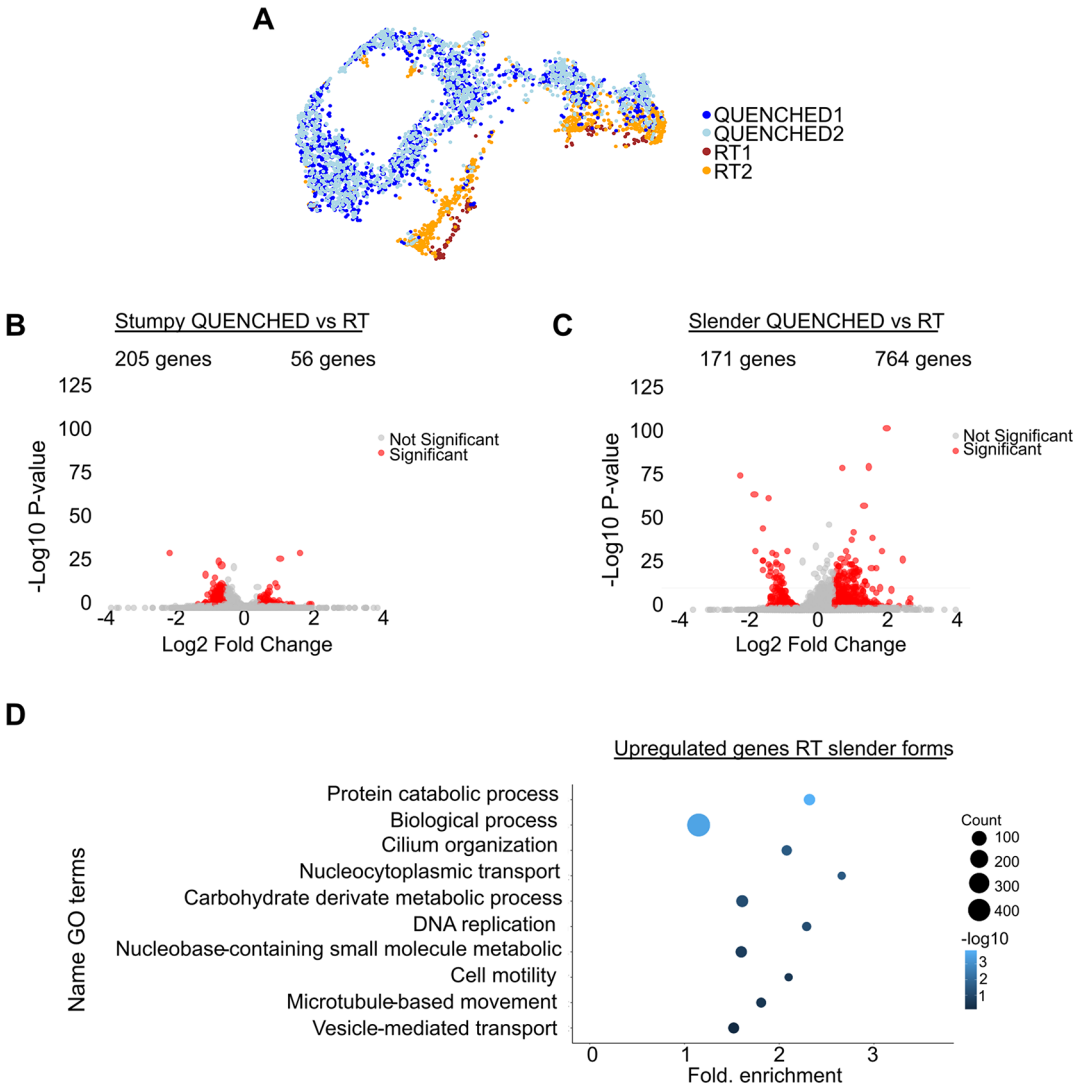

Figure 3. The transcriptome of slender forms is more sensitive to temperature variations than stumpy forms. (A) Uniform Manifold Approximation and Projection (UMAP) plot of four merged samples: in blue the QUENCHED samples and in orange/brown the RT samples. (B) Volcano plot of differentially expressed (DE) genes of stumpy forms in QUENCHED versus RT conditions. Genes colored in red are significantly differentially expressed. (C) Volcano plots of differentially expressed (DE) genes of slender forms in QUENCHED versus RT conditions. Genes colored in red are significantly differentially expressed. (D) Gene ontology (GO) analysis of differentially expressed genes upregulated in slender form (panel B) of RT condition. The color of the dots represents the adjusted p value of each GO term: from dark blue (higher) to light blue (lower). The size of the dot represents the gene count in each GO term, ranging from the smallest dot (lowest count) to the largest dot (highest count).

slender forms in QUENCHED versus RT conditions (see Table S3). We identified a total of seven genes associated with temperature shock (0.7% of all significantly regulated genes): four were upregulated under the QUENCHED condition and three were downregulated. Therefore, we conclude that neither condition experienced more shock-related expression than the other.

In summary, quickly dropping temperature does not have a major impact on transcriptome of slender forms, and even less on stumpy forms. Not surprisingly, in slender forms, quick cooling reduced the transcript levels of a few genes involved in parasite motility and proliferation.

Discussion

Over the past decade, single-cell RNA sequencing (scRNA-seq) methods have been significantly improved. Various single-cell isolation techniques, such as fluorescence-activated cell sorting (FACS),[36] microfluidic droplets,[5] and

emerging technologies like PARSE[37] (which utilizes cell labelling and multi-pipetting) and BD Rhapsody[38] (which employs microwells), have been developed. While these methods are effective, they are optimized for standard mammalian cells. In this paper we describe that quenching of parasites prior to microfluidics-based encapsulation (such as in 10x Genomics), immobilized parasites, helping with the cell recovery and avoiding major transcriptomic stress.

Protocols from 10x Genomics have been successfully used in *T. brucei*,[26,32,39] often incorporating an ice incubation step prior to encapsulation, which makes the parasites more immotile than normal RT. However, significant loss of parasites—larger than typical mammalian cells—has been observed in several studies, with recovery rates ranging from 20% to 52% (22% in this study) while in mammalian cells recovery rates vary between 40% to 65%.[33,40] While other factors can be involved (size of the cell, for instance) we hypothesized that cell motility could be also involved.

Placing parasites on ice reduces their motility. However, because encapsulation happens at room temperature, parasites may partially recover their motility. In fact, previous studies using microfluidic have shown that *T. brucei* parasites survive and even divide for extended periods within droplets.[41] In this study we confirmed that encapsulating parasites at room temperature using the 10x Genomics microfluidic device does not induce changes in motility or morphology (Supplementary Videos 1–2 extended data).

In this study, we observed a proportion of approximately 80:20% slender to stumpy forms in the QENCHED condition. This differs from our previous study in 2016,[30] where we detected a proportion of ~14% slender forms and ~86% stumpy forms. Other studies have also reported varying proportions of stumpy and slender forms at day 6 post-infection, ranging from 22% to 39% slender forms.[28,42] It is important to note that these previous studies classified parasites based on PAD1 expression and morphology. In the present study, we classified cells using a combination of different gene expression markers (see Methods), as PAD1 was not detected in our dataset, likely due to technical limitations already noted in other scRNA-seq studies.[26,32] While PAD1[43] is typically expressed upregulated in early-stage stumpy differentiation, PAD2[43] and EP1[44] (two of the gene used for stumpy classification here) are more characteristic of late-stage stumpy forms and procyclics.[45,46] Due to these differences in classification criteria, (as well as normal variation between animals in the proportion of slender and stumpy forms), it is not surprising that we observed a lower proportion of stumpy forms compared to previous studies.

To reduce the chances that parasites would not recover their motility during encapsulation, while avoiding transcriptomic changes due to cold shock, in this work we decided to quench parasites (rapid cooling), a method traditionally used in metabolomics studies to reduce artifact changes associated to cold shock.[42] The only difference between quenching parasites or placing them on ice (standard protocol recommended by 10x Genomics), is the rapid cooling of the parasite to 0°C on ethanol in a dry ice bath (as explained in the Methods section). Parasites are never frozen, and after 5–7 seconds on this bath, the tube is transferred to ice to keep parasites cold. While we did not compare quenching versus standard ice protocol side by side, by comparing our efficiency of cell recovery with published data, it seems the efficiencies are similar. Quenching has already been used in bulk RNA-seq in *T. brucei* to reduce cold shock.[43]

While analysing the transcriptomic data, we detected an interesting pattern that could be helpful to distinguish *T. brucei* life cycles stages with different levels of VSG expression. Indeed, we found that stumpy forms displayed a higher number of genes and transcripts per cell than slender forms. This is likely due to the differential expression of VSGs between these two life cycle stages. VSG mRNA is highly abundant in slender forms and thus many reads (7.5% of total reads in this dataset) align to this single gene, reducing the opportunity for other transcripts to be sequenced. When VSG expression is lower, other transcripts become more detectable, resulting in the detection of more genes/cell. This effect could explain why in other scRNA-seq studies of *T. brucei* tsetse fly stages, non-VSG-expressing stages such as procyclic forms and epimastigotes have a higher number of transcripts and genes per cell than VSG-expressing metacyclic forms (between 10%–50% higher).[32,39,44] This finding could aid in the identifications of cell clusters in *T. brucei* scRNA-seq datasets.

While we did not find major transcriptomic differences between RT and QUENCHED conditions (Figure S2), 10% of genes were differentially expressed in the slender forms between the two temperature conditions (Figure 3A). Specifically, at RT we found upregulation of genes involved in motility and proliferation, and importantly we did not detect changes in gene expression that would suggest cells were under stress (Figure 3D). These results are consistent with the observations shown in the Supplementary Videos 1–4, in which the quenched parasites do not exhibit any morphological or behavioural changes.

While the data shows that when parasites were incubated at RT the capture efficiency was lower than when parasites were quenched, we cannot unequivocally attribute this to the encapsulation moment, as we were unable to observe the process continuously with a camera. Additionally, the loss of these parasites could be due to lower data quality for unknown

reasons related to temperature. Using a lower cutoff for the number of genes per cell could increase cell recovery, but it is difficult to determine whether these are real cells or ambient RNA, and further research is needed. However, we believe it is important to consider the implications of motility when working with highly motile organisms. Previous studies describing scRNA-seq on *Leishmania*[45] or *Trypanosoma cruzi*[46] have consistently included a step in which parasites were placed on ice prior to encapsulation, which seems to at least partially mitigate motility. These consideration should be taken into account in microfluidics-based analysis of other highly motile unicellular organisms, such as *Chlamydomonas reinhardtii*,[47] *Euglena gracilis*,[48] some Diatoms[49] and bacteria as *Pseudomonas aeruginosa*.[50]

Exploring how scRNA-seq microfluidic devices selectively capture distinct cell populations within a heterogeneous sample is crucial for understanding the limitations and biases of these methods. Such studies could lead to refining and optimizing scRNA-seq protocols, enabling more efficient accurate capture of cellular diversity across different biological contexts.

Ethical approval

All animal experiments were conducted in accordance with European Union regulations (Directive 2010/63/EU) and approved by the Órgão Responsável pelo Bem-Estar Animal (ORBEA) of Instituto de Medicina Molecular and the Direcção Geral de Alimentação e Veterinária (DGAV) under licenses 018889/2016 (approved on 26/08/2016) and 017549/2021 (approved on 20/10/2021). All efforts were made to minimize suffering, and humane endpoints were established. Animals were monitored daily for signs of distress, and humane euthanasia was performed when necessary. Handling was kept to a minimum to reduce stress, and all procedures prioritized animal well-being.

Declaration of generative Ai and Ai-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT 4o in order to improve the readability of the text originally written by the team. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Data availability

Underlying data

The raw FASTQ files from the scRNA-seq data are available in the NCBI Sequence Read Archive (SRA) under the project title *"Cell motility influences the encapsulation process in scRNA-seq microfluidics"* with accession number PRJNA1227990. The dataset can be accessed at: https://www.ncbi.nlm.nih.gov/sra/PRJNA1227990.[51]

Extended data

The extended data is in Zenodo under the title "Cell motility influences microfluidics capturing in scRNA-seq", https://zenodo.org/records/17712924[52]

This project contains the following underlying data:

Document with the supplementary legend

- Supplementary Figure and Video Legends.docx

Seurat object with the processed data:

- InVivo_RT_QUENCHED.rds

All supplementary tables:

- TableS1.xlsx
- TableS2.xlsx
- TableS3.xlsx

All supplementary videos:

- Video 1. avi
- Video 2. avi
- Video 3. avi
- Video 4. avi

All supplementary figures:

- FigureS1.tiff
- FigureS2.tiff

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Reporting guidelines

Zenodo: Laritabonita/Encapsulation: Codes, 10.5281/zenodo.15194552[53]

ARRIVE checklist. https://doi.org/10.5281/zenodo.15194552[53]

Software availability

The analysis in this article was performed using RStudio (R version 4.4.1), an open-source software (https://posit.co/download/rstudio-desktop/), licensed under the GNU Affero General Public License (AGPL) version 3. The source code is available from https://github.com/Laritabonita/Encapsulation under the DOI https://doi.org/10.5281/zenodo.17712924.[52] This dataset is made available under a Creative Commons Attribution 4.0 International License (CC BY 4.0), permitting unrestricted use, distribution, and reproduction, provided the original work is properly cited.

All the code used in this paper can be found under the DOI https://doi.org/10.5281/zenodo.15194552[53]

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The videos were collected using the 3i Slidebook Reader v.6.0.22 (https://www.intelligent-imaging.com/slidebook), but for those without access to 3i Slidebook, equivalent acquisition analyses can be performed using open-source alternatives such as ImageJ (https://imagej.net/ij/), which is also used for video analysis and is available under a Public Domain license.

Author contributions

TG: Formal Analysis, Writing – Review Editing.

LG: Funding Acquisition, Supervision.

LLE: Writing-original draft, designed the study, carried out all experiments and data processing, analysed the data, wrote the manuscript.

LMF: acquired funding, designed the study, supervised the project, and edited the paper.

AN: carried out microscopy visualization experiments.

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Open Peer Review

Current Peer Review Status: ? ? ✓

Version 2

Reviewer Report 10 April 2026

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✓ **Virginia Howick**
University of Glasgow, Glasgow, Scotland, UK

The authors have addressed all my comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Parasitology, single-cell genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 28 August 2025

https://doi.org/10.21956/openreseurope.21399.r55289

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? **Virginia Howick**
University of Glasgow, Glasgow, Scotland, UK

López-Escobar et al have provided an important study on how cell motility influences capture efficiency in T. brucei blood stages. They compared samples that were run on the 10X genomics platform at room temperature to samples from the same infection that were quenched to 0 degrees C. They found that quenching improves retention of the more motile

slender form in their dataset. Generally, the paper is very clear and well presented and the method will be broadly useful to researchers using single-cell microfluidics platforms on motile and other atypical cell-types. Although the study is well designed and the results are presented clearly, I'm not fully convinced that the increased recovery of slender forms is due to reduced cell motility.

Major comment
QC thresholds impact on impact on proportion of stumpy vs slender
I am somewhat concerned that the loss of the slender forms in the RT condition could be due to different levels of gene expression between the two cell types and a decrease in cell quality generally, not just in slender forms. If you have lower genes per cell across samples in slender forms (as they mostly express VSG), and then you apply a high QC threshold, you will bias the sample towards removing those cells. Even if that is the case, the protocol is still useful, it just has less to do with cell motility, and more to do with overall cell quality. To help clarify this, you could include the QC plots of genes and UMI per cell for each sample (like fig S1) in order to visualise where the cut off (800 genes per cell) falls on the distribution. Does it seem like there are viable cells being removed unnecessarily? Additionally, you could visualise the genes per cell on the UMAP to see if this breaks down by cell type and by condition (RT vs quenched). Are the quenched stumpy forms also expressing fewer genes per cell in the RT condition compared to quenched? This would make me think the quenching is more a general improvement compared to motile-specific improvement. Finally, if you use a lower cut off (eg 300 genes per cell), how does that change the proportion of cell-types recovered?

Minor comments
DE analysis
A low cluster resolution was used to distinguish slender and stumpy forms; however, for the DE it might give more insight if you increased the cluster resolution to isolate the slender cluster that is majority RT cells. Within that cluster what is DE between RT and quenched? I realise the cell numbers from the quenched samples in the cluster might be too low, but worth a try.

Sequence saturation
I don't completely follow the explanation that VSG is over saturating the sequencing in slender forms and this is a technical issue. Because 10X sequences all cells together after transcripts have their UMI and barcode, wouldn't abundant VSG expression also decrease the number of transcripts detected in stumpy forms? Or do you think that the barcodes/UMIs within the GEM are limited? Alternatively, could it be a limitation on the cell machinery? It would be helpful to understand how deeply your samples were sequenced, i.e. did you reach sequencing saturation?

Page 7, first paragraph: There is no supplementary video 5 provided.

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Parasitology, single-cell genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 28 August 2025

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?

Igor Cestari
McGill University, Montreal, Canada

This is a very well-written and nice report by López-Escobar and colleagues. The work reports single-cell experiments to improve the capture of T. brucei cells using a low-temperature fast quenching vs. room temperature and comparing results from slender vs. stumpy parasite forms. The work will be very useful in guiding experiments on T. brucei single-cell assays using microfluidics. The protocol could apply to other flagellated cells, and although differences may still exist between different cell types, the experience shared in this work will be extremely useful.

I noted a few points below for the authors, and I hope they take these suggestions/questions to improve the manuscript:

1) In Table 1. Gene/cell, is this an average of all cells? Can you please indicate the variation? This will help evaluate the degree of noise and how it changes after filtering for cells with low gene counts. The same applies to the other columns.

2) Fig 2. How does the distribution of quenched 80%:20% slender: stumpy relate to the initial distribution of slender to stumpy? How can we know that 80:20 is the expected ratio? Although the variation is lower in the quenched than in RT, it does not determine the correct distribution of slender:stumpy in the population. Having the input information would be ideal. Hence, an independent measure should be used, such as microscopic counting or flow cytometry, or perhaps another information collected by the authors, to ensure that the collected values by quenching the samples are representative of the population and if any deviation, the authors could point it out.

3) To my point above, the authors concluded: "quenching probably facilitated the recovery of less motile slender forms within the GEMs and thus the final sequenced population of parasites is more representative of the original population in the blood." Provide some support for this.

4) Differences in RT1 and RT2 are very difficult to see in Fig 3A. Can you change the colours for better visualization?

5) I wonder how many of these cells in the population represent dying cells. I am not sure if there is a specific marker that could be used, as it would be interesting to know the state of the cells. Dying cells will also have a transcriptome, but how this could affect the overall population analysis and thus add bias to experiments is unclear. Perhaps, cell cycle markers should be included in the analysis, such as those analyzed in 2C, to identify the cell cycle stages and perhaps abnormalities that could be indicative.

6) Does the lower right population (~4 o'clock in cluster 0) in Fig. 2A represent typical live cells in any cell cycle stage, or could those be dying cells? Why are they forming such a distinct population?

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Parasitology, molecular and cellular biology, genomics, proteomics, genetics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 27 June 2025

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General Comments
This manuscript addresses an important and often overlooked technical limitation in single-cell RNA sequencing of motile organisms. The study is well-designed and timely, particularly for researchers working with kinetoplastids and other highly motile cells. The proposed protocol modification (rapid cooling or "quenching") is clearly described and convincingly tested. I believe the paper makes a valuable contribution to the scRNA-seq methodology literature. However, several clarifications and improvements in writing and data presentation would enhance the clarity and rigor of the work.

General Suggestions
- Please consider depositing the processed data as a .Rds file in Zenodo as well, this will greatly improve transparency and reproducibility. Apologies if I missed it, but I couldn't find it among the other code and .Rds files.
- In the supplementary file with figure legends, include legends for the supplementary videos as well. These videos are valuable to the reader, but currently lack adequate description.

Points for Clarification

1. The authors state that earlier *T. brucei* scRNA-seq studies (e.g., references 26, 28, 32 - also 44?) achieved equal or better cell recovery rates using protocols that kept parasites on ice before 10x encapsulation. Given this, the added value of the quenched protocols described here are, of course, its ability to improve representation and reduce capture bias, rather than simply

increasing overall cell recovery.
In this regard, the authors present compelling evidence - based largely on scRNA-seq data - that quenching enhances the representation of certain subpopulations that might otherwise be underrepresented. However, the conclusions would be strengthened by including an external validation step (pre- and post- encapsulation), such as microscopy-based quantification or RT-qPCR using stage-specific markers, to confirm that the cell-type proportions observed after quenching more accurately reflect those found in vivo.
2. In the UMAP of merged, non-normalized RT and quenched samples, subclusters are visible within both the slender and stumpy populations. Since the authors state that no integration or batch correction method was applied to address potential technical variation, I suggest applying such methods, and maybe assess clustering stability. This could help determine whether the observed substructures reflect true biological variation or are artifacts of technical differences. Additionally, differential expression analysis between subclusters and evaluation of known marker expression could provide further insights into their biological relevance.
3. The methods section suggests that the authors performed unsupervised clustering, followed by annotation using marker genes from Briggs et al. (2021). Could the authors confirm whether this was a fully unsupervised approach, or if any supervised step as reclassification based on marker expression was included at any point? This clarification is especially important given the observed substructure within slender and stumpy populations in the merged UMAPs. If the clustering was truly unsupervised, additional analyses, such as testing marker gene expression across subclusters or comparing clustering outcomes with and without integration, could help evaluate whether these structures reflect biological subtypes, technical batch effects, etc.
4. In the section *"Quenching improves retention of slender forms"*, the final paragraph restates earlier points and could be deleted entirely without loss of clarity.
5. This may be a matter of writing style, but I found the directionality in the GO term discussion somewhat confusing. While the differential expression is performed as QUENCHED vs RT, the GO enrichment results are discussed as if referring to RT vs QUENCHED, without explicitly indicating the reversal. For instance, GO terms enriched in genes downregulated in QUENCHED are described as features of RT conditions. To improve clarity and help the reader follow the interpretation more easily, it might be helpful to align the direction of the discussion with the DE analysis as performed or briefly note when the direction is being inverted for narrative purposes.
6. While the authors report no significant enrichment of stress-related GO terms, stress-responsive changes can be subtle or missed by broad GO categories. Could they include a targeted analysis of known heat-shock or cold-shock genes to more sensitively assess whether quenching induces physiological stress?

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to

ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Parasitology, Molecular biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.