

INTRODUCTION

In flow cytometry, fluidic instability can occur during sample acquisition and lead to artifacts in the data. Unstable profiles are of variable form and magnitude (**Figure 1**).

In a large-scale operation, it can prove time consuming and difficult to manually exclude unstable regions and to stay consistent between flow cytometry data analysts and across several months or years of a clinical study.

Thus, to efficiently generate homogeneous and reliable clinical trial data, flowCut [1, 2], an R-based package developed for the automated fluidic instability clean-up, was evaluated, then included into Caprion's analysis pipeline.

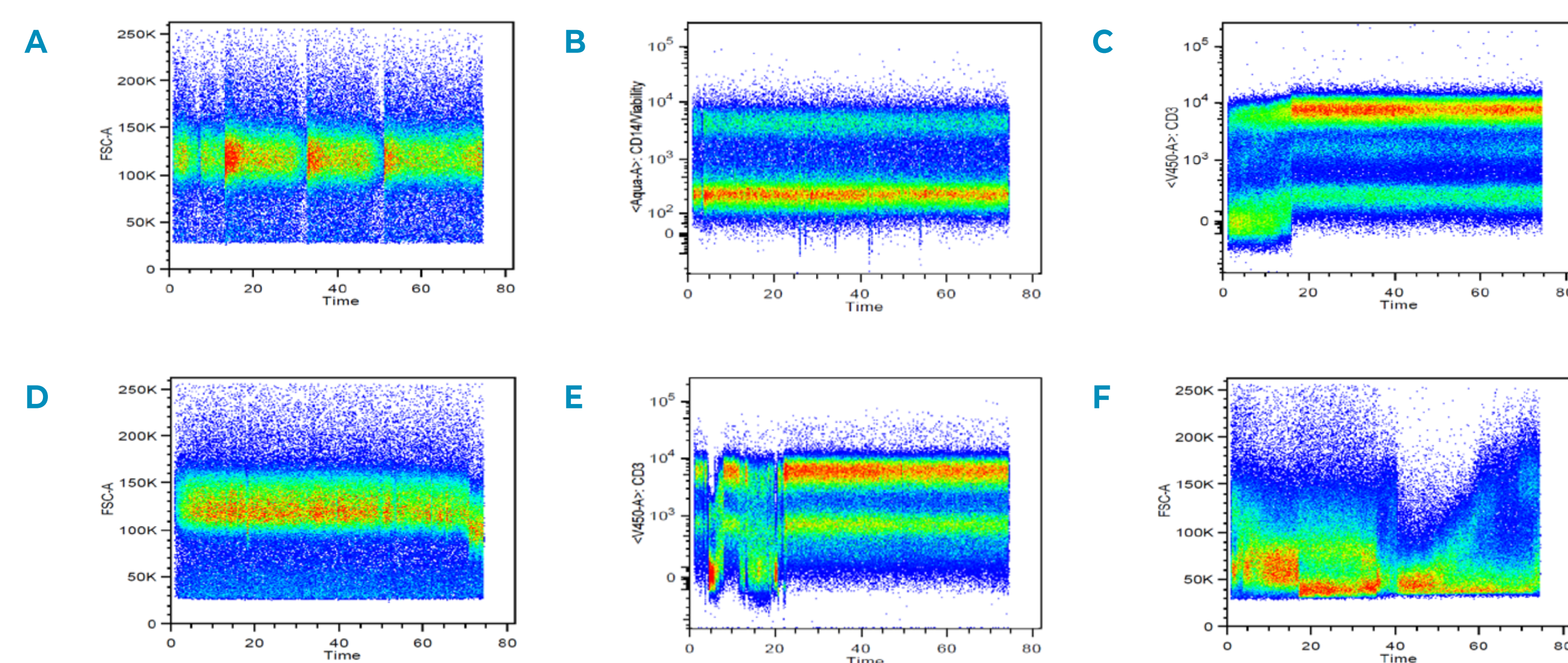


Figure 1. Examples of fluidic instability. Fluidic types can include but are not limited to: short breaks (A), fast subsequent decreases in specific channels (B), fast signal increase (C) or decrease (D), as well as other more major issues such as wave-like signal (E) and complete signal breakdown (F).

METHODS

FLOWCUT ALGORITHM DESCRIPTION:

To assess outlying fluorescence patterns, the flowCut algorithm segments the sample across time and verifies which segments are different from the others. Its evaluation is based on the variation of the fluorescence signal across segments, using inputs such as the mean, median, percentiles, variance, and skewness.

Built-in parameters can be adjusted such as segment size, and cut-off or sample flag thresholds. For example, if the segment size is set too high, small fluidic instabilities may be missed, whereas if it is set too low, stable segments could get discarded due to a higher normal variation between them.

FLOWCUT ALGORITHM FINE-TUNING:

To ensure the flowCut algorithm reflected the manual decisions typically made by expert flow cytometry data analysts, an array of FCS files containing different types of fluidic instabilities was used to optimize selected built-in parameters:

- From their default value, parameters were modified, one by one, to evaluate their impact on fluidic cleansing.
- Parameters were then changed simultaneously: out of more than 50 different combinations, the one that mimicked the most manual gating choices was selected.

To further fine-tune the parameters, several experiments containing clinical study samples were tested. They contained or not major fluidic instability, and they represented different sample matrices, such as fresh whole blood, frozen PBMCs and tumor samples.

FLOWCUT SYSTEM DESIGN:

The flowCut system, which was built around the flowCut algorithm, was designed to allow a robust high-throughput semi-automated fluidic clean-up solution that can be used in conjunction with any other standard flow cytometry gating software.

• Fluidic Outliers Parameter

Instead of directly removing outliers from the FCS files following the flowCut processing, the flowCut action is instead embedded in a new parameter inside the processed FCS file (**Figure 2**). This allows the analyst to discard or complement the flowCut processing if needs be.

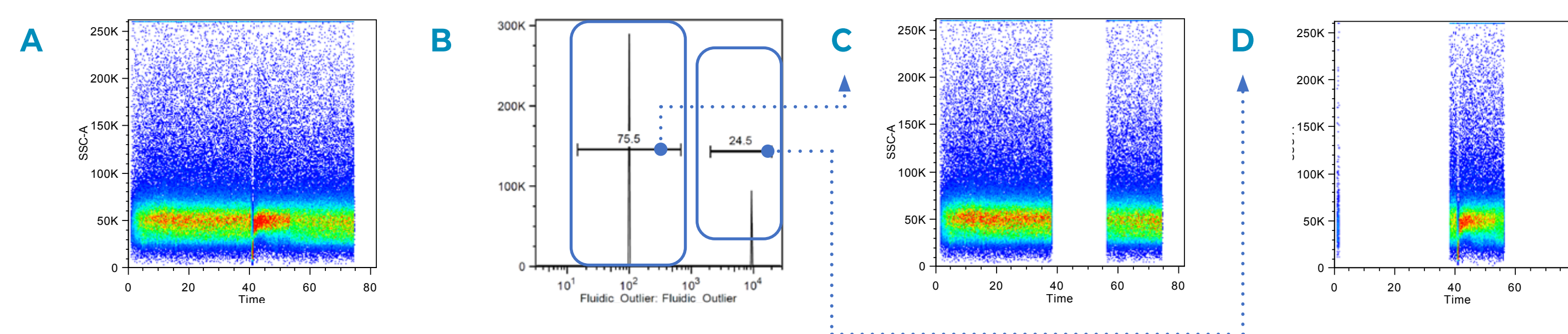


Figure 2. Each event is tagged in a new parameter within the processed FCS File. Time vs SSC-A dotplot of a sample with fluidic instability, Ungated (A). Histogram representation of the new Fluidic Outliers parameter (B). Time vs SSC-A dotplot of events categorized as fluidic outliers (D) or not (C).

• PDF Report

The PDF reports associated with each experimental run processed by flowCut was enhanced to facilitate its review by flow cytometry data analysts, and flag samples requiring further attention based on fluorescence signal continuity.

• Scheduler

A task scheduler was developed to process newly acquired FCS files automatically at pre-defined times each day. This ensures that the FCS files are ready promptly for subsequent data analysis (**Figure 3**).

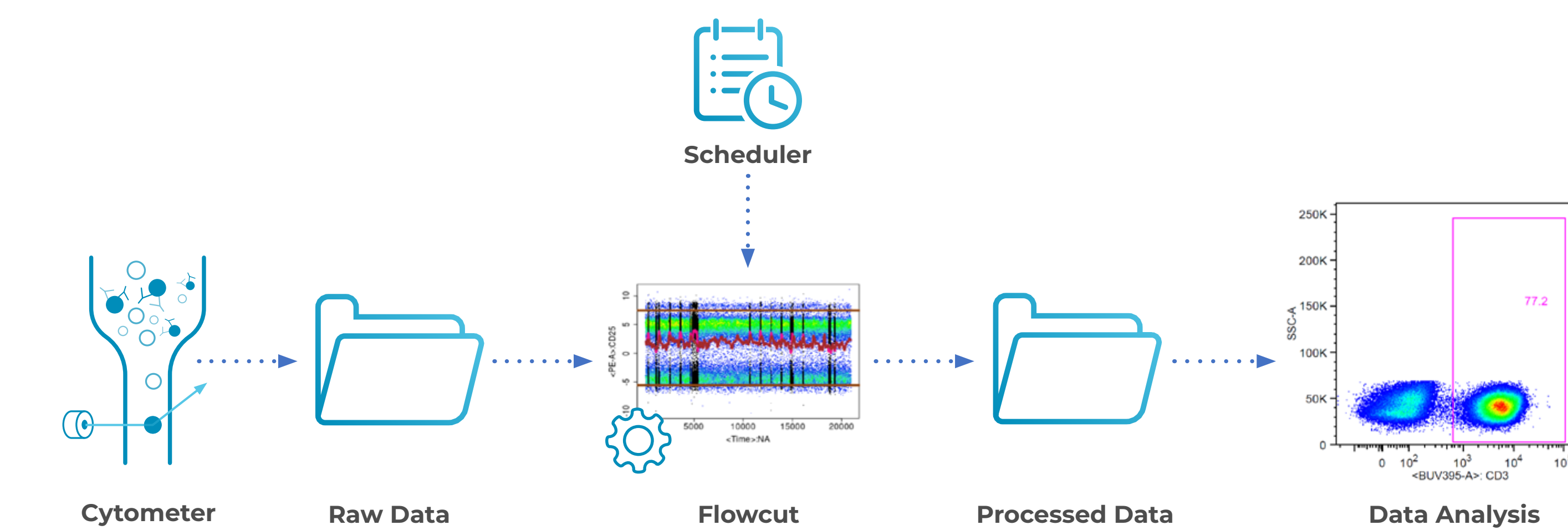


Figure 3. Analysis Pipeline with flowCut. Following FCS files transfer from cytometer to Raw Data file share, the task scheduler, at pre-defined times, automatically selects new experiments to process with the flowCut algorithm. The processed FCS files and PDF report are placed in a different file share to allow subsequent analysis.

• Controlled Environment

Both the flowCut system installation and the resulting processed data are stored in a centralized and controlled environment which is protected from unintentional alteration by end users. The system installation was validated and is maintained using a rigorous change control process.

RESULTS

Given that very small segments can be removed and since the removal process does not depend on the number of exclusion gates that are part of the existing gating strategy, as with manual gating, flowCut automated fluidic cleansing is more precise and targeted than what a flow cytometry data analyst is able to do (**Figure 4**).

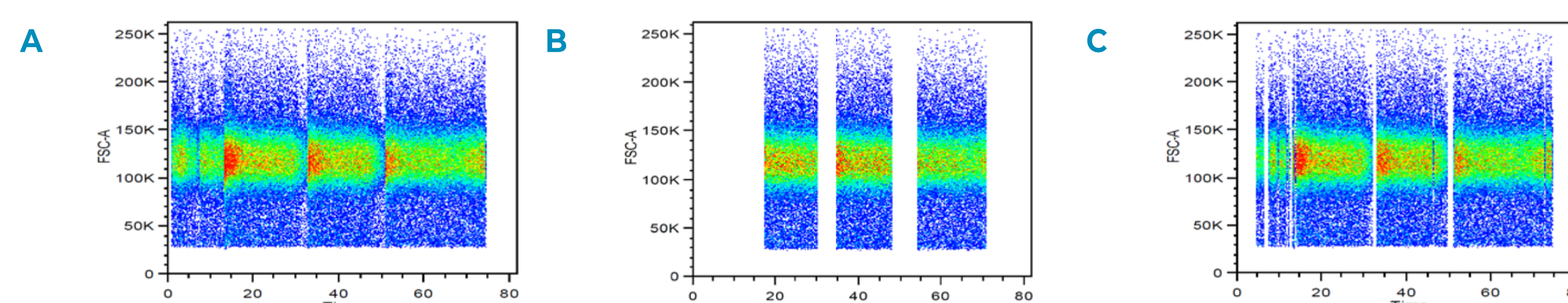


Figure 4. Automated gating is more precise than manual fluidic instability exclusion. Example of a sample with fluidic instability: time vs FSC-A: ungated (A), gated after the manual fluidic instability exclusion (B), gated after the automated cleansing performed by the flowCut algorithm (C).

Although the automated clean-up is consistent and robust, it will always try to keep the longest continuous signal, which can lead to the removal of the wrong segment in rare cases. In fact, what is seen as a small perturbation, but really is not, will be excluded over a lengthy inappropriate signal. These issues can only be discovered by comparing fluorescence signals between technical replicates of the same sample, a capability that is not part of the current flowCut algorithm. In these cases, the flow cytometry data analyst review is critical to correct the automated choice made by flowCut (**Figure 5**).

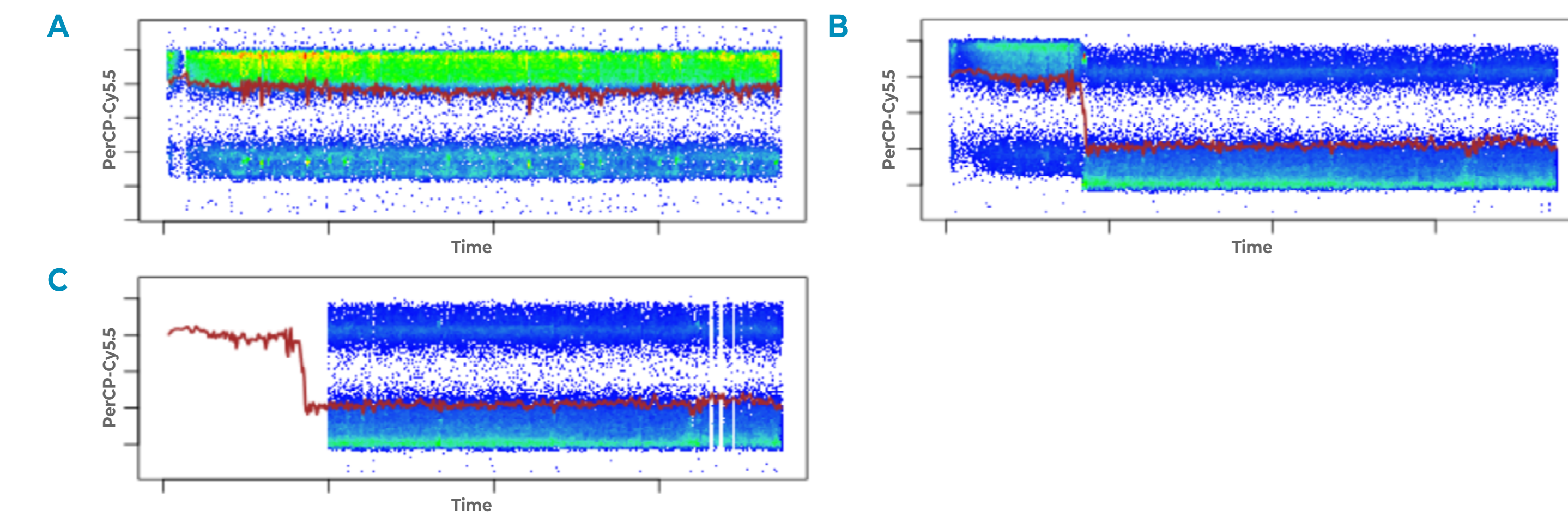


Figure 5. In rare cases, the wrong part of the sample can get excluded. Example from flowCut PDF report of two technical replicates of a same sample: time vs PerCP-Cy5.5, ungated, replicate #1 and #2 (A-B). Replicate #2 is shown after flowCut processing (C).

DISCUSSION

Through an extensive design and testing, the flowCut System provides an efficient and robust fluidic cleansing of the FCS files. Its validation was also performed to ensure its compliance to required regulations. Furthermore, compared to manual fluidic instability exclusion, the fluidic is cleansed more accurately and, since the same parameters are applied to every experiment, the inter-analyst variability is removed. Moreover, the PDF reports generated by the flowCut system are still reviewed by expert flow cytometry data analysts with the ability to complement flowCut cleansing, ensuring that the highest standards are maintained.

CONCLUSION

Manual gating can remove unstable signals but is time consuming and difficult to standardize among different flow cytometry data analysts. In a high-throughput operation, the use of a semi-automated fluidic instability cleansing solution preserving the scientific expertise will not only lead to efficiency gains but will also improve the robustness of the data.

References: 1. Justin Meskas and Sherrie Wang (2019), flowCut: Precise and Accurate Automated Removal of Outlier Events and Flagging of Files Based on Time Versus Fluorescence Analysis. R package version 0.99.11. 2. Meskas J, Wang S, Brinkman R. 2020. flowCut — An R package for precise and accurate automated removal of outlier events and flagging of files based on time versus fluorescence analysis. bioRxiv doi: 10.1101/2020.04.23.058545.