Flow cytometry and stroke: from current methodology to future applications

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Flow cytometry is a versatile technique for analyzing stroke-induced changes in the immune system. Unlike other methods of cell identification such as immunohistochemistry, the technique is rapid, highly sensitive, and capable of quantifying multiple markers in cell suspensions. Utilizing cell sorters, flow cytometers can also produce highly enriched populations of viable cells for functional studies. The aim of this perspective is to appraise current flow cytometry methods in the field of stroke, provide guidance on best practices, and outline some of the future applications of the technique in pre-clinical and clinical stroke research.

The immune response during acute ischemic stroke: The immune system is involved in all stages of acute ischemic stroke, from risk factors to tissue repair (Malone et al., 2019a). Post-cerebral ischemia, a series of events involving the brain, the vasculature, the blood, and lymphoid organs are invoked. Dying cells release damage-associated molecular patterns, activating resident microglia. The resulting pro-inflammatory environment, coupled with increased blood-brain barrier permeability, promotes immune cell entry into the infarct site. Brain-invading leukocytes secrete collagenases, gelatinases, reactive oxidative species, cytokines and leukotrienes, producing further neurotoxicity. Ultimately, brain inflammation is self-limiting and gives way in the subacute and chronic stages of stroke to structural remodeling and functional reorganization. Local and infiltrating immune cells are also involved in this reconstruction and repair. Already, there are many protocols for both the isolation of immune cells from brain and subsequent analysis via flow cytometry.

Isolating immune cells from the post-ischemic brain for flow cytometric analysis: The key steps in cell isolation from the brain are mechanical dissociation of tissue, separating the resulting cell suspension using discontinuous (30/70%) Percoll gradients by centrifugation, and then collecting leukocytes at the interphase (Pino and Cardona, 2011).

Within the stroke field, Campanella et al. (2002) offered a slight modification through addition of a 37% density layer, yielding a cleaner separation. Möller et al. (2012) used a 21/38/80% system for the same reason. Both Pösel et al. (2016) and Ballesteros et al. (2014) performed single-layer density gradient procedures to cut down on the time-consuming preparation of gradients. Evidently, while multi-layer separations may prove beneficial for downstream sorting applications, because of practicality and reproducibility, simple discontinuous gradients remain widely used (Chu et al., 2014).

While there remain some disagreements on the best method of density centrifugation, other aspects of published cell isolation protocols show less variation. Before removal of the rodent brain, the ascending aorta should be perfused with saline, thus removing blood from the cerebral vasculature (Pösel et al., 2016). In this way, only leukocytes that have extravagated into the brain parenchyma are analyzed. Omission of this step results in an overestimation of infiltrating leukocytes by ~10–20% (Chu et al., 2014). Conversely, inefficient mechanical dissociation of brain tissue or lack of digestion buffers (e.g. DNase + collagenase I/II) may reduce viable immune cell yield (Pösel et al., 2016). However, while DNase can be included as a means of reducing cell clumping, care should be taken when employing collagenases due to lot-to-lot variability and potential surface antigen cleavage (Campanella et al., 2002; Pino and Cardona, 2011).

Experimental design variables affecting the quantification of the immune response in stroke: *A priori* power calculations should be conducted to determine the number of animals required to observe significant changes in immune cell parameters of interest. These calculations should include data from pilot studies or peer-reviewed publications. The data should also reveal the expected frequency of the target population within the target tissue, which will inform the number of cells that must be stained and acquired.

In order to maximize the numbers of target immune cells obtained for flow cytometric analysis, attention should be paid to the two factors which most influence leukocyte infiltration into the infarcted brain, namely the model of brain ischemia and the time since ischemia onset (Grønberg et al., 2013). Across all models of middle cerebral artery occlusion (MCAO), the trend is for the peak infiltration of neutrophils to occur between days 1–3, macrophages between days 3–7, while lymphocytes accumulate more gradually. However, as early as 5 days post-ischemia, substantially higher numbers of T and B cells can be seen in permanent MCAO as opposed to transient MCAO (Zhou et al., 2013). Differences in microglial activation and the expression of inflammatory markers (e.g. interleukin-1, tumor necrosis factor-α, interferon-γ, and intracellular adhesion molecule-1) are also noted. Overall, independent of infarct size, 50% fewer infiltrating leukocytes are observed at 24 hours post-transient MCAO compared with permanent MCAO (Chu et al., 2014). In the photothrombotic model of stroke, where a multi-vessel permanent occlusion is produced, enhanced numbers of brain-infiltrating lymphocytes can likewise be seen in the subacute stages of ischemia compared with transient MCAO. Notably, however, a recent systematic review of post-ischemic immune cell brain infiltration shows that both the duration (transient *vs.* permanent) and location (proximal *vs.* distal) of vessel occlusion influence the extent of leukocyte entry (Beuker et al., 2021). Crucially, the same review concludes that, despite established differences in blood leukocyte composition between rodents and humans, the temporal dynamics of post-ischemic neuroinflammation are comparable. Of all the leukocytes included, only macrophages show relatively lower levels in human stroke lesions compared with rodents in the early post-stroke phase, and even this difference may be explained by the semi-quantitative nature of the histological staining employed in human studies. Indeed, in rodent samples, flow cytometry is also able to confirm an initial wave of macrophage infiltration not apparent in histological analysis. Overall, this demonstrates the role flow cytometry can play in quantifying the immune response to stroke and its superiority to immunohistochemistry for detecting small changes in immune cell infiltration during the acute post-stroke period.

Separately, the time since ischemia onset should be a prime consideration for those using flow cytometry to determine

immune cell activation, function and phenotype. In the case of resident microglia, for example, activation occurs within minutes of brain ischemia, providing a key opportunity to analyze proliferation and intracellular cytokine staining via flow cytometry in the acute period (Malone et al., 2019b). The gradual shift from an anti-inflammatory "M2" (e.g. CD206$^+$) phenotype towards a pro-inflammatory "M1" (e.g. CD68$^+$) phenotype among microglia in the first week post-ischemia, on the other hand, informs the selection of markers for flow cytometry panels. The combined effects of stroke model and time since ischemia onset perhaps have the most impact on the quantification of subpopulations such as regulatory T cells (Tregs). In permanent MCAO, these cells may represent up to 20% of CD4$^+$ cells infiltrating in the early post-ischemic period (Malone et al., 2021). In transient MCAO, conversely, substantial Treg invasion may not occur until weeks post-ischemia. In both models consideration may be given to pooling tissue in order to isolate sufficient Tregs for accurate analysis.

Flow cytometry variables affecting the quantification of the immune response in stroke: Despite widespread use, as a method flow cytometry remains highly variable, especially with regards to sample handling, reagents, instrument setup, and data analysis. Only within the last decade has there been a concerted movement towards standardized immunophenotyping in human clinical studies. But effective flow cytometric analysis in stroke relies not only on standard antibody panels. It requires careful and consistent sample preparation, a well-defined staining protocol, validated reagents, a calibrated instrument, and the inclusion of several experimental controls (Cossarizza et al., 2019). All antibodies in the staining panel should be used at a pre-determined titration specific for the cell type and assay conditions. If using the same antibody to study cells in multiple tissues in stroke, however, aspects including antibody concentration, incubation temperature, incubation time, and number of cells will need to be re-validated. In terms of instrumentation, rigorous quality control encompasses optimization of photomultiplier tube voltages, fluidics stability, and spectral compensation. On the analysis side, data should be screened for fluctuations in fluid dynamics, and then cleaned of doublets and dead cells. Proper controls are required to confirm the antibody specificity for the staining target. Cells which do not express the marker

of interest can be employed, although these are often difficult to obtain. In such scenarios, the inclusion of reagents which block "sticky" Fc receptors on monocytes, macrophages, dendritic cells, and B cells should at least reduce false positives and increase the discrimination between cells which are positive or negative for the antigen of interest. Isotype controls can be included to verify the success of this strategy. Controls are also required to positively identify the cells expressing the antigen of interest through the process of "gating." Unstained samples are not sufficient to determine the difference between the background autofluorescence emitted by cells and a "true" positive signal. As a result, many researchers once again turn to isotype controls. However, isotype controls assume all unspecific staining is due to the isotype of the antibody. In fact, positive staining with isotype controls usually indicates antibody binding to cell FC receptors, an interaction that as noted above can be easily blocked. In fluorescence minus one controls all antibody conjugates in the experiment are included except the antibody being controlled for. This provides a robust measure of fluorescence spread from other antibodies into the channel of interest. In all multiparameter staining protocols, fluorescence minus one controls should be included as the gold standard (Cossarizza et al., 2019). In cytokine assays, unstimulated cells should be included as an added control due to non-negligible basal expression.

Reporting flow cytometry data in the stroke literature: To ensure pre-clinical and clinical stroke results are reproducible, full details of protocols also need to be published. In 2008 the "Minimum Information about a Flow Cytometry Experiment (MIFlowCyt)" guidelines were proposed (Lee et al., 2008). Using a checklist approach, these guidelines outline the information researchers should provide in relation to samples, reagents, instrument configuration, and data analysis. For flow cytometry antibodies, the supplier, catalogue number, analyte, clone, and fluorochrome should be included. For analysis, a detailed gating strategy is paramount so that, for instance, other researchers can see that dead cells were excluded, or determine whether a CD4$^+$ percentage represents a frequency of a CD3$^+$ parent or of total lymphocytes. Finally, researchers should be encouraged to deposit raw data in repositories in order to promote systematic reviews of stroke candidate drugs, allow for multicenter clinical trials, and enhance our overall

understanding of the immune component of the disease.

Future applications of flow cytometry in the field of stroke: While progress on the accurate reporting of flow cytometric data in the stroke literature has at times been slow, the development of new applications for the technique has accelerated. The availability of cytometers capable of measuring dozens of parameters has prompted the development of dimensionality reduction tools which allow for the visualization of high-dimensionality data in a lower-dimensional space (Cossarizza et al., 2019). Non-linear methods such as t-stochastic nature embedding offer intuitive representations of large datasets, while newer algorithms such as uniform manifold approximation and projection (UMAP) scale better and provide improved global structure. Using a combination of large antibody panels and UMAP, clinical stroke researchers have mapped immune alterations in the early post-stroke period, with a view to following up on novel phenotypes which track with disease outcome (Krishnan et al., 2021). Ultimately, this may allow us to determine which cells can act as biomarkers for short-term outcomes like infection, or even act as predictors for longer-term sequelae such as cognitive decline and depression. Separately, flow cytometry is providing a means to study both the mechanisms and the extent of more complex phenomena such as stroke-induced immunodepression. Flow cytometry will also likely be at the heart of attempts to combine information at a cellular level with emerging data on the role genetics play in post-stroke inflammation. Finally, the advent of mass cytometry provides a novel method of comprehensively assessing the functional state of the immune system post-stroke (Cossarizza et al., 2019).

In conclusion, the combination of flow cytometry and preclinical animal models of brain ischemia has provided both a broader and a deeper picture of the multifaceted role of immune cells in stroke progression (Malone et al., 2019a). However, at both preclinical and clinical levels, issues with sample handling, reagent choice, instrument setup, data analysis, and result reporting remain (**Figure 1**). The application of advanced flow cytometry techniques such as mass cytometry may narrow the gap between preclinical and clinical studies. Ultimately, given the versatility of flow cytometry in quantifying immune cell frequency, phenotype, cytokine expression, and

Workflow Stage	Key Considerations
1. Experimental Design	• Determine number of samples required for flow cytometric analysis via power calculations. • Model of brain ischemia (transient *vs.* permanent, proximal *vs.* distal). • Optimal timepoint post-ischemia for isolation of cell type of interest from infarcted brain.
2. Cell Isolation	• Flush blood from cerebral vasculature to remove non-extravasated immune cells. • Digestion of brain tissue (mechanical *vs.* chemical). • Percoll gradients (single-layer *vs.* discontinuous).
3. Antibody Staining	• Use standard antibody panels at pre-determined titrations and incubation conditions. • Include Fc receptor blocking reagents and viability dyes. • Include fluorescence minus one (FMO) controls (+ unstimulated cells for cytokine assays).
4. Sample Acquisition	• Optimize photomultiplier tube voltages. Confirm fluidics stability. • Use appropriate single-colour compensation controls. • Acquire enough events to detect target population.
5. Data Analysis	• Inspect fluid dynamics. Exclude doublets and dead cells. • Use FMO controls to discriminate between negative and positive populations. • Use dimensionality reduction techniques (t-SNE, UMAP) for exploratory investigations of high-dimen

6. Data Reporting	• Report results according to the MIFlowCyt guidelines:
	  • Include detailed gating strategy.
	  • Include antibody supplier, catalogue number, analyte, clone, and fluorochrome.
	  • Deposit raw data in data repositories.

Figure 1 │ Key considerations for researchers using flow cytometry to quantify the immune response to stroke at each stage of the experimental design (yellow), data collection (green), and analysis and dissemination (blue) process.
t-SNE: t-stochastic nature embedding; UMAP: uniform manifold approximation and projection.

protein phosphorylation, the technique will undoubtedly remain a primary means to study the immunopathogenesis of stroke, assess the clinical response to treatment, and aid in the development of immunotherapies which could enhance functional recovery for stroke survivors worldwide.

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