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UFM^{Track}: Under-Flow Migration Tracker enabling analysis of the entire multi-step immune cell extravasation cascade across the blood-brain barrier in microfluidic devices

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Abstract

The endothelial blood-brain barrier (BBB) strictly controls immune cell trafficking into the central nervous system (CNS). In neuroinflammatory diseases such as multiple sclerosis, this tight control is, however, disturbed, leading to immune cell infiltration into the CNS. The development of in vitro models of the BBB combined with microfluidic devices has advanced our understanding of the cellular and molecular mechanisms mediating the multi-step T-cell extravasation across the BBB. A major bottleneck of these in vitro studies is the absence of a robust and automated pipeline suitable for analyzing and quantifying the sequential interaction steps of different immune cell subsets with the BBB under physiological flow in vitro.

Here we present the Under-Flow Migration Tracker (UFM^{Track}) framework and a pipeline built based on it to study the entire multi-step extravasation cascade of immune cells across brain microvascular endothelial cells under physiological flow in vitro. UFM^{Track} achieves 90% track reconstruction efficiency and allows for scaling due to the reduction of the analysis cost and by eliminating experimenter bias. This allowed for an in-depth analysis of all behavioral regimes involved in the multi-step immune cell extravasation cascade. The study summarizes how UFM^{Track} can be employed to delineate the interactions of CD4⁺ and CD8⁺ T cells with the BBB under physiological flow. We also demonstrate its applicability to the other BBB models, showcasing broader applicability of the developed framework to a range of immune cell-endothelial monolayer interaction studies. The UFM^{Track} framework along with the generated datasets is publicly available in the corresponding repositories.

Author summary

Immune cells continuously travel through our body to perform immune surveillance. They travel within blood vessels at a very high speed and slow down upon reaching their target

organ by the sequential interaction with different adhesion and signaling molecules on the vascular endothelial cells.

The study of molecular mechanisms mediating this multi-step extravasation of immune cells has been significantly advanced by *in vitro* cultures of microvascular endothelial cell monolayers. The dynamic interaction of the immune cells with endothelial monolayers can be imaged over time *in vitro* in microfluidic devices under physiological flow. The 2-dimensional structure of the endothelial monolayer allows for reliable visualization of the extravasation process required for the study of the molecular mechanisms involved. The manual analysis of the acquired imaging data is time-consuming and prone to experimenter error. Analysis automation is, however, hampered by the similar appearance of the unlabeled immune and endothelial cells and by the flow causing rapid immune cell displacement.

Here we introduce ^{UFM}Track, the under-flow migration tracker framework allowing for automated analysis of immune cell interactions with microvascular endothelial cells under flow *in vitro*. ^{UFM}Track performs comparably to the manual analysis of an experienced researcher, eliminates experimenter's bias, and improves the accuracy of the immune cell tracking. Taken together, ^{UFM}Track sets the stage for scalability of *in vitro* live cell imaging studies of immune cell extravasation.

eLife Assessment

This work is **important** because it elucidates how immune cells migrate across the blood brain barrier. In the revised version of this study, the authors present a **convincing** framework to visualize, recognize and track the movement of different immune cells across primary human and mouse brain microvascular endothelial cells without the need for fluorescence-based imaging using microfluidic devices. This work will be of interest to the cancer biology, immunology and medical therapeutics fields.

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Introduction

Immune cells continuously travel throughout our body as a means of immune surveillance. Moving within the bloodstream allows for their fast transport to even distant sites but requires extravasation once they have reached their target organ. Immune cell extravasation across the vascular wall is a multi-step process regulated by the sequential interaction of different signaling and adhesion molecules on the endothelium and the immune cells (1 [↗](#), 2 [↗](#)). These molecular interactions mediate distinct sequential steps, namely tethering and rolling to reduce travel speed, shear-resistant arrest, polarization and crawling of the immune cell on the luminal surface of the endothelium, and finally, immune cell diapedesis across the endothelial layer (2 [↗](#)–4 [↗](#)).

The precise molecular mechanisms mediating the multi-step immune cell extravasation in each organ depend on the immune cell subset but also the specific characteristics of the vascular bed. For example, in the central nervous system, the endothelial blood-brain barrier (BBB) establishes a tight barrier that strictly controls the transport of molecules across the BBB, ensuring tissue homeostasis required for neuronal function (2 [↗](#), 5 [↗](#)). The BBB similarly controls immune cell trafficking into the CNS. Thus, accounting for these special barrier properties, unique characteristics of the multi-step T-cell migration across the BBB have been described. For instance,

T cells crawl for very long distances against the direction of blood flow on the surface of the BBB endothelium in search of permissive locations for diapedesis (6–8). Research on T-cell interaction with the BBB has already been successfully translated into therapies in the clinic (9,10).

Exploring the entire multi-step extravasation of immune cells across the BBB has been significantly advanced by making use of in vitro BBB models maintaining their barrier properties, and placing them into microfluidic devices (11). Combined with microscopic setups that allow for in vitro live-cell imaging (Figure 1A–B) of the immune cell interaction with the brain endothelial monolayer under physiological flow over time, the molecular mechanisms mediating the sequential interaction of T cells during extravasation across the BBB have been delineated (2). The live cell imaging is largely performed in the phase-contrast imaging modality (Figure 1C), which does not require establishing fluorescent labels of the imaged cells and avoids potential photo-toxicity of the fluorescent imaging modality. These studies have shown that upon their arrest, T cells polarize and either crawl at speeds between 3 to 10 $\mu\text{m}/\text{minute}$ over the brain endothelial monolayer or probe the endothelial monolayer by remaining rather stationary and sending cellular protrusions into the endothelial monolayer (Figure 1D) (12,13). Both behaviors can lead to diapedesis of the T cells across the brain endothelial monolayers. This process lasts at least 3 to 5 minutes, with some immune cells observed to protrude and retract several times prior to finalizing a prolonged diapedesis process to the abluminal side of the brain endothelial monolayer (8,14). Finally, T cells that have successfully migrated across the brain endothelial monolayer usually continue to migrate underneath the endothelial monolayer (15,16).

The data analysis of these “in vitro flow assays” requires time-consuming offline frame-by-frame analysis of the imaging data by individual experimenters, in which the dynamic interactions of each individual immune cell has to be followed over the entire time of the assay manually and assigned to specific categories. Such analysis is tedious, and accurately assigning the different T-cell behaviors requires experience. Thus, this manual analysis is prone to inevitable errors and subjective judgments of the various experiments. Furthermore, the time-consuming manual T-cell tracking limits the number of events that can be studied and, thus, the statistical power of the analysis.

Automation of the analysis of the recorded multi-step T-cell extravasation across the BBB in the microfluidic device would thus be highly desirable. It is, however, hampered as these assays are usually performed with unlabeled cells and imaged by phase contrast (Figure 1C), which poses a challenge due to the similar grayscales and morphology of the immune cells interacting with the brain endothelial cells. Further challenges include perfused on top T cells that, in the presence of shear flow, instantly appear within the field of view (FoV) and are either suddenly displaced over a certain distance or completely detached and washed away (Figure 1D). Proper analysis of these events is mandatory for reliable T-cell tracking but also with respect to the study of the overall avidity of the dynamic T-cell interaction steps with the underlying brain endothelium. Thus, it was compulsory to establish a tracking solution that accounts for the effect of the flow on the migrating cells and the distinct migration regimes.

Segmenting of cells imaged in phase contrast migrating on top of cellular monolayer of similar appearance is a challenging task. Furthermore, the analysis of T cell interactions requires detection and quantification of the transmigration. While recent advances in computer vision established several frameworks for detection (17–20) or segmentation (21,22), no tool is readily available to complete both tasks simultaneously.

There is a large number of powerful cell tracking solutions developed that focus on different aspects of cell migration, and are embedded in different frameworks or solutions. The general-purpose Trackpy (23) allows for simple particle tracking and is not suitable to track cells

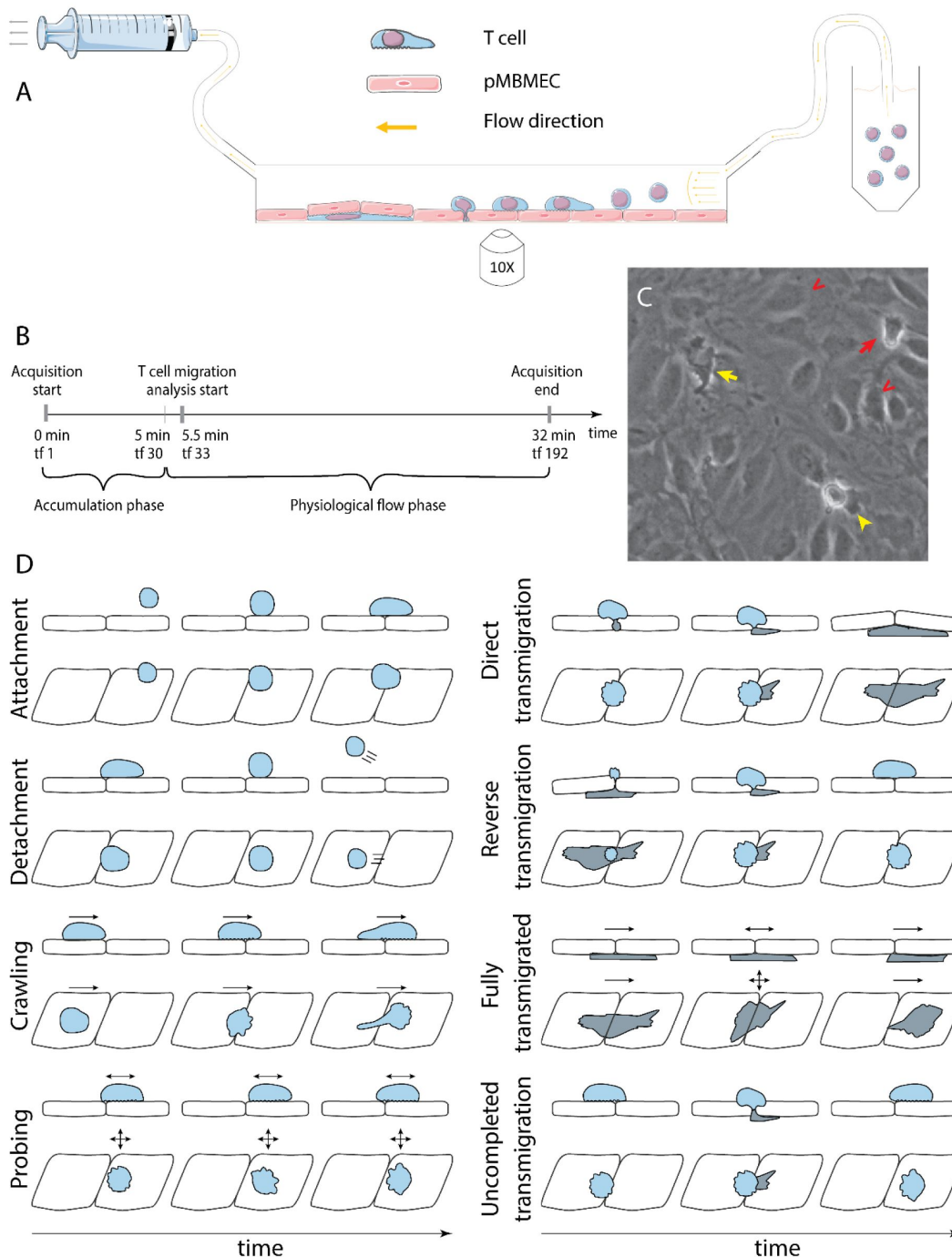


Figure 1.

In vitro analysis of the multistep cascade of T-cell migration across the BBB model under physiological flow. **A.** In vitro under-flow assay setup. T cells were perfused on top of the pMBMEC monolayer, and their migration under flow was observed using phase-contrast imaging modality. Imaging was performed with a time step of 10 seconds/timeframe. **B.** In vitro flow assay timeline. During the accumulation phase under flow with the shear stress of 0.1 dynes/cm^2 , T cells adhered to the pMBMEC monolayer. After 5 minutes (timeframe 30), the shear stress was increased to 1.5 dynes/cm^2 , leading to rapid detachment of not firmly adhering T cells. Analysis of the post-arrest T-cell behavior was thus starting at 5.5 minutes (timeframe 33). tf = timeframe. **C.** Example of phase-contrast imaging data. Red arrow – crawling T cell; yellow arrow – fully transmigrated T cell; yellow arrow-head – transmigrated part of a partially transmigrated T cell; red V arrowheads – pMBMECs. **D.** Schematic representation of distinct T-cell behavior regimes detected and analyzed using the developed ^{UFM}Track framework. Crawling cells migrate continuously while probing cells interact with the pMBMECs and move around the interaction point within two cell-size ($20 \mu\text{m}$) as indicated by the arrows. Side and top views are shown.

migrating under flow. It is used for the tracking of cells imaged in phase contrast in the Usiigaci framework (24 [↗](#)). The widely used TrackMate (25 [↗](#)) plugin for ImageJ (26 [↗](#)) allows for tracking of particles performing either the Brownian motion or a linear motion, but not the combination of two. Instant appearance or disappearance as well as the resolving tracks of under-segmented cells cannot be easily established in this framework. Similarly, CellTraxx (27 [↗](#)) performs matching of cells in the adjacent frames, prohibiting its use for tracking of under-segmented, intersecting cells or under flow. The Bayesian Tracker (btrack) framework (28 [↗](#)) is using spatial information as well as appearance information for track linking focusing on the cell divisions required for cell lineage tracing.

Here we introduce the developed under-flow migration tracker (UFM Track) framework that systematically addresses the hurdles mentioned above, allowing it to perform automated tracking and analysis of cell-cell interactions imaged in phase contrast. We also show a successful implementation of UFM Track to build an analysis pipeline for T-cell interactions with brain microvascular endothelial cells in vitro under physiological flow. UFM Track reaches 90% T-cell tracking efficiency, performing comparably to manual analysis while eliminating the experimenter's bias and improving the accuracy of T-cell tracking. Therefore, it enables significant savings in the labor force and time for data analysis.

Results

To design and develop the automated T-cell under-flow migration analysis framework UFM Track framework presented here, we made use of in vitro imaging datasets following T-cell migration across primary mouse brain microvascular endothelial cells (pMBMECs) under physiological flow in vitro. The framework combines three components: T-cell segmentation and transmigration detection, T-cell tracking under flow, and analysis of each of the multistep T-cell migration cascade steps. Segmentation and transmigration detection of the T cells, migrating on the pMBMECs, is performed with a 2D+T U-Net-like convolutional neural network (21 [↗](#)). The T-cell under-flow tracking algorithm was formulated as a constrained optimization problem.

Next, we describe the methods and algorithms employed to develop UFM Track. Links to the code and the datasets used for model training can be found in the Data Availability section.

I. T-cell segmentation

Reliable cell segmentation is crucial for reliable cell tracking. In the phase-contrast imaging modality, it was impossible to achieve reliable differentiation between T cells and endothelial cells of the pMBMEC monolayer based on pixel intensity. For detection of T-cell transmigration across pMBMEC monolayers (diapedesis), sufficiently reliable cell segmentation of the transmigrated T cells was also required. To achieve this, we have designed 2D and 2D+T U-Net-like (21 [↗](#)) fully convolutional neural network-based models for multitask learning. The models were trained to predict three maps: cell probability, the probability that the T cell is below the pMBMEC monolayer, and cell centroids. The models performed predictions based on the grayscale of the respective phase-contrast images in the case of the 2D model or sequences of 5 timeframes in the case of the 2D+T model. The models were implemented in TensorFlow (29 [↗](#)). The training was performed on a dataset corresponding to 154 Megapixels of raw image data using the annotation mask for T cells ("T cell mask") and the mask of the transmigrated part of the T cells ("transmigration mask"), the centroids map, and the weight map (Figure 2 [↗](#)). For validation 37 Megapixels of raw imaging data were used. The models have approximately 3M and 8M parameters correspondingly, and the model architectures are summarized in Supplementary Tables 1, 2. Details on models' implementation, training, and image processing can be found in the "Segmentation models" section of the Supplementary Material.

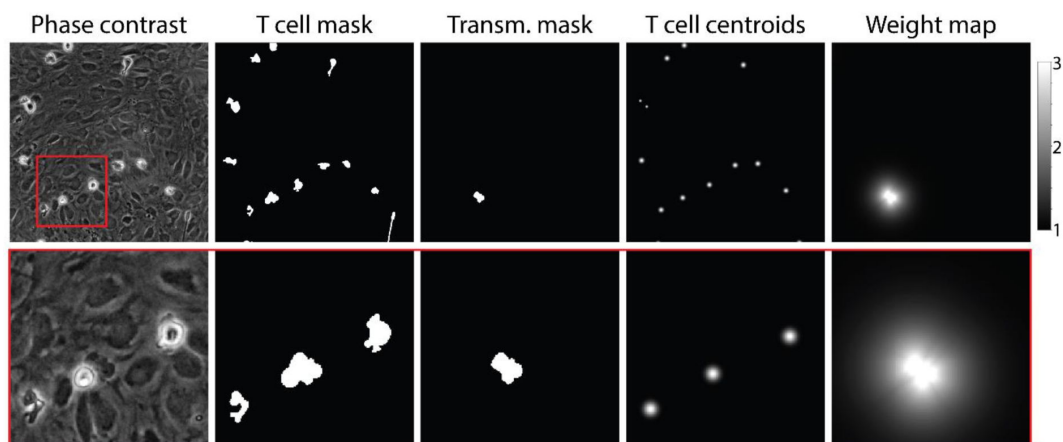


Figure 2.

Example of data used for training of the T-cell segmentation model. From left to right: Phase-contrast microscopy image of T cells migrating on top of the pMBMEC monolayer; annotated T cell mask; annotated mask of transmigrated part of T cells; T cell centroids; w_{cell} weight map. The bottom row shows zoom-in on the highlighted area.

In the T cell mask prediction task, the 2D+T model outperformed the 2D model by a notable 9% according to the average precision (AP) metric (**Table 1**). At the same time, for the transmigration mask, which is much more difficult to infer, the 2D model performance reached only 54%, which is insufficient for reliable detection of T cells migrating across the pMBMEC monolayer. In this task, the 2D+T model outperformed the 2D model by 32% AP. We observed that the 2D+T model was sensitive to frame misalignment, leading to false positive detection of transmigrated T cells. Thus, to generate the preliminary T-cell masks used for frame alignment and histogram normalization, we employed the 2D model. Afterward, for the T-cell segmentation and transmigration detection, we employed the 2D+T model, followed by a watershed algorithm based on the inferred T-cell mask and the cell centroids as seed points. A stitched phase-contrast image sequence can be seen in Supplementary Video 1 and overlaid with the segmented cells and highlighted transmigration mask in Supplementary Video 2.

After segmentation, we suppressed the noise by discarding small objects with an area below 30 pixels (the mean area of a T cell is about 400 pixels). For each T cell, we evaluated its coordinates

as geometric mean coordinate, angle of the longer axis, and elongation $\epsilon = \sqrt{\frac{\sum_i (x'_i - x'_0)^2}{\sum_i (y'_i - y'_0)^2}}$, where x'_i ,

y'_i are projections of the i -th T cell mask pixel coordinates on the orthogonal long and short axes of the T cell, and x'_0, y'_0 are the corresponding projections of the geometrical center of the T cell. The summation is performed over the area covered by the mask of the corresponding T cell.

Additionally, we evaluated the mean T cell probability $\overline{p_{cell}} = \frac{1}{n_{pix}} \sum_i p_{cell,i}$, mean

transmigration probability $\overline{p_{tr}} = \frac{1}{n_{pix}} \sum_i p_{tr,i}$, and cell transmigration coefficient as

$t_c = \frac{1}{n_{pix}} \sum_i \frac{\min(p_{tr,i}, p_{cell,i})}{p_{cell,i}}$, where n_{pix} is number of cell pixels and $p_{cell,i}, p_{tr,i}$ are the T cell and transmigration probabilities for i -th pixel in the predicted T cell mask.

II. T-cell tracking

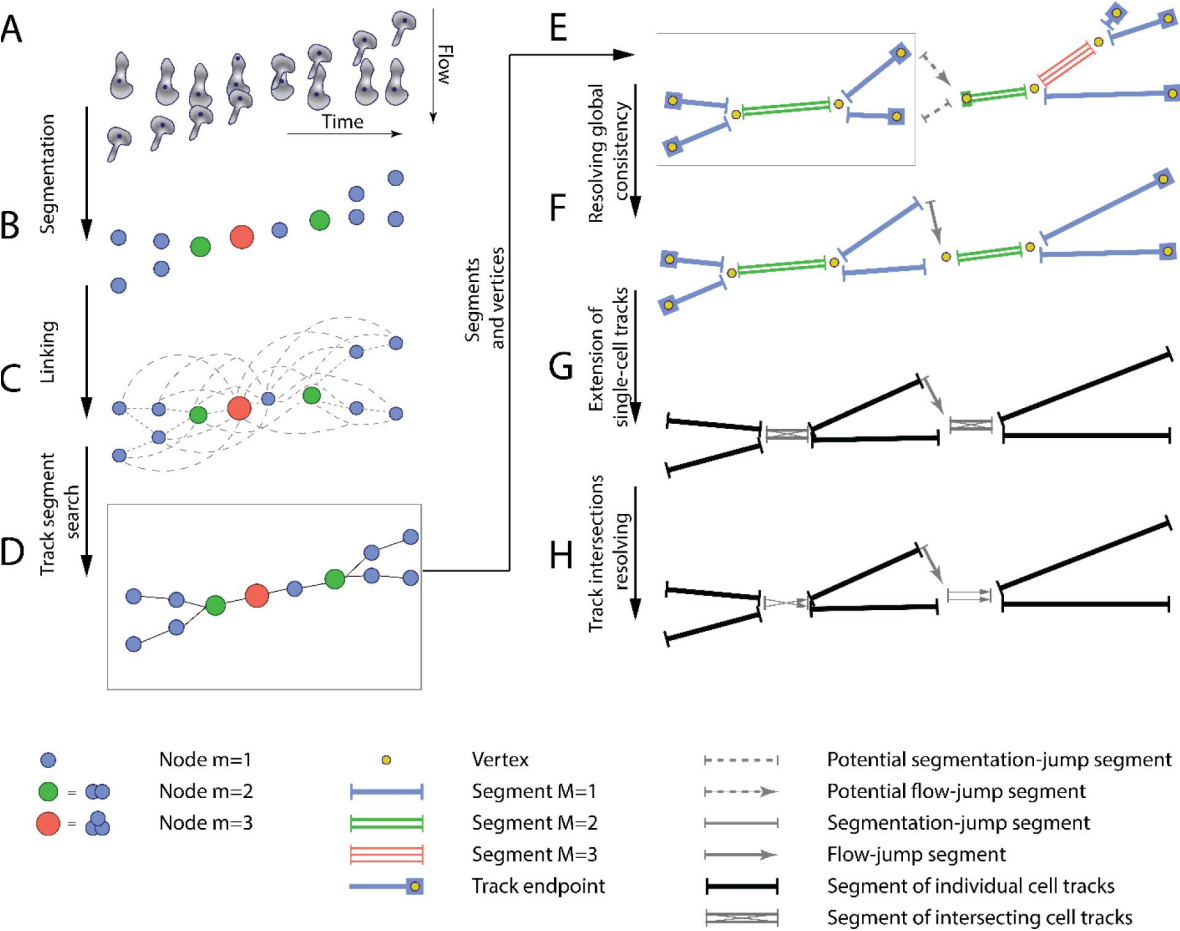
The presence of flow causes several types of discontinuous events that were managed with a specialized tracking algorithm. These are: 1) the sudden appearance of T cells in the field of view (FoV), 2) the sudden detachment of a T cell followed by its disappearance from FoV, and 3) the displacement over significant distances (up to hundreds of micrometers) of T cells that do not adhere firmly to the pMBMECs. The primary inspiration for our approach was the Conservation Tracking algorithm (30). We could consistently reconstruct the T-cell tracks by performing global optimization constrained to controlled probabilities of T-cell appearance, disappearance, and displacement due to the flow at every time point. The standard tracking algorithms like tracking by association is either unable to detect track links with high displacement and thus reconstructs several disconnected segments of a cell track, or leads to a high number of false track links. Our procedure favors the reconstruction of long T-cell tracks while at the same time allowing for tracking of the T cells which detach or are displaced by the flow (“accelerated T-cell movement”) over a longer distance ($> 8 \mu m$) with speed significantly higher than their crawling speed.

The tracking consists of four main steps: linking, searching for track candidates, global track consistency resolving, and resolving the track intersections. The inputs for the tracking are the segmented cells and the cell centroids (**Figure 3A**). The centroids and the cell proximity are used to quantify under-segmentation by introducing the node multiplicity (**Figure 3B**, see section T-cell tracking of the supplementary information for details). At the linking step, we identified all possible connections of T cells between the timeframes (**Figure 3C**).

Table 1.
Comparison of the performance of the 2D and 2D+T-cell segmentation models.

	Performance, AP	
	2D	2D+T
T Cell mask	86.12%	95.26%
Transmigration mask	50.56%	82.74%

Figure 3.
The T-cell tracking pipeline. A. Segmentation and centroids of two migrating T cells under flow over time. B. Nodes corresponding to segmented T cells. Node sizes and colors denote the multiplicity estimate of a node. C. Nodes, together with links between adjacent in space and time nodes, form a graph. D. Selected links are obtained with global optimization on the graph. E. Graph of segments and vertices obtained according to node multiplicity. F. Resolving global segment multiplicity consistency with global optimization on the graph. Additionally, a search for rapid displacement of segmented T cells due to under-segmentation or flow is performed. G. Extension of tracks of individual T cells into the track intersection. H. Resolving track intersections.



Next, during the search for track candidates, we aimed to find continuous track segments of T cells or under-segmented groups of T cells crawling on top of or below the pMBMEC monolayer without accelerated movement segments on the T cell track characterized by rapid T cell displacements. The whole dataset of T cells across all time points was represented as a graph. Each vertex corresponds to a T cell (multiplicity $m=1$) or a group of potentially under-segmented T cells ($m>1$). The vertices are connected according to links obtained at the linking step. Track segments were found by performing global optimization to find consistent connectivity of vertices across the time points (**Figure 3D**) by employing an approach inspired by the conservation tracking algorithm summarized in Schiegg et al. (30). Optimization was performed using the CP-SAT constrained optimization procedure using the open source OR-Tools library (31).

Next, resolving global track consistency was performed. In this step, the scope of T cell tracking is shifted from individual nodes (representing T cells at particular timeframes as well as groups of under-segmented T cells) to the track segments (unambiguous sequences of nodes) and vertices at the endpoints of the segments. These are track start and end points, points of merging and separation of track segments in case of under-segmentation, as well as ambiguous points on a track. The latter was identified by sudden T cell displacement, a hallmark of detaching and reattaching T cells and T cells transitioning from properly segmented to under-segmented T cells or vice versa (**Figure 3E**). This was followed by the search for potential missing track segments due to accelerated T cell movement under flow and missing links in the track crossing points. Both are characterized by significant displacement lengths such that they were not detected during the linking step. Thus we will refer to both as “jumps” in this section. We have also eliminated short and therefore unreliable segments, as well as segments whose multiplicity was found to be $M = 0$ (**Figure 3F**). Afterward, we separated the segments into two categories, namely segments with multiplicity $M = 1$, i.e., tracks of isolated T cells, and segments with multiplicity $M > 1$, i.e., tracks of under-segmented groups of T cells, where tracks of several T cells intersected. Finally, we extended the tracks of isolated T cells into the intersection if the branching vertex had a multiplicity of n and was splitting in exactly n segments with multiplicity $M = 1$ (**Figure 3G**).

Lastly, we resolved the track intersections to obtain reliable T cell tracks, i.e., identifying track segments corresponding to the same T cell before and after the under-segmented track region (**Figure 3H**).

Detailed information on the developed under-flow T cell tracking algorithm is given in the “T-cell tracking” section of the Supplementary Material.

III. T-cell migration analysis

Next, we performed the T-cell migration analysis based on the reconstructed T cell tracks. We selected tracks inside the fiducial area of the FoV, namely coordinates of the T cell at all timepoints along the track were located at least 25 μm away from the bounding box enclosing all segmented T cells. Next, tracks of T cells touching another T cell at the end of the assay acquisition were excluded since T cells directly adjacent to each other can hide the start of T cell transmigration across the pMBMEC monolayer and thus compromise correct detection and quantification of this step. Additionally, only tracks with T cells were assigned in at least 6 timeframes during the physiological flow phase. We also require T cells to be assigned for at least 75% of timeframes along the track. Under-segmented parts of T cell tracks were not considered. Examples of selected tracks can be seen in Supplementary Video 3.

After exclusion of the tracks of the cellular debris, which were misclassified as T cells during the segmentation step, using a dedicated “not-a-T-cell” classifier, we performed identification of transmigration (Supplementary Figure 5), probing and crawling migration regimes, as well as accelerated movement along the T cell track. Detailed information on the analysis of the T cell tracks is given in the “T-cell migration analysis” section of the Supplementary Material.

Finally, for each track, we evaluated motility parameters for each of the following migration regimes: probing before the transmigration, crawling before the transmigration, all crawling above pMBMECs monolayer including T cell crawling segments after the first transmigration attempts, all crawling below pMBMECs monolayer, whole T-cell track excluding accelerated movement and tracking inefficiency regions, as well as whole T-cell track. Specifically, we evaluated the following T-cell motility parameters: duration of each migration regime, the total vector and absolute displacements, the migration path length, the average migration speed (displacement over time), average crawling speed (path length over time), and finally the mean and standard deviation of the instantaneous speed. We evaluated migration time, displacement, and average speed for the accelerated movement regime.

IV. Analysis of trafficking datasets

Having developed a full pipeline based on the ^{UFM}Track framework for automated analysis of the multi-step T-cell migration cascade across the BBB model, we next aimed to compare the results of the automated analysis with previous studies, assess the capacity of the framework to gain novel insight into T-cell migration under flow, and evaluate its performance as compared to manual analysis.

1. CD4⁺ T cell analysis

To this end, we first analyzed a total of 18 imaging datasets each corresponding to 32 minutes recording dedicated to understanding the multi-step migration of CD4⁺ T cells across non-stimulated (n= 6), TNF stimulated (n=5), and IL1- β -stimulated (n=7) pMBMEC monolayers under physiological flow with the developed pipeline.

In these in vitro live cell imaging datasets, phase-contrast imaging was performed as described in the "Data acquisition" subsection in the Materials and Methods section. The T-cell accumulation phase corresponding to the shear stress of 0.1 dynes/cm² lasted for 32 timeframes (5 minutes), followed by conditions of physiological flow (shear stress 1.5 dynes/cm²) for the subsequent 160 timeframes (27 minutes). The shear stress is controlled by an automated syringe pump. After increasing to physiological flow rates ([12](#) [14](#) [32](#)), a significant number of T cells detached from the pMBMEC monolayers, with higher numbers of T cells detaching from non-stimulated pMBMEC monolayers when compared to those stimulated with pro-inflammatory cytokines as detected by the distribution of the track ending times between 5 and 30 minutes. (**Figure 4A** [12](#) [14](#) [32](#)).

Analyzing these datasets with the established ^{UFM}Track framework, the T-cell behavior type for each detected T-cell track and the aggregated T-cell behavior statistic were obtained for the stimulated and non-stimulated pMBMEC monolayers (**Figure 4B** [12](#) [14](#) [32](#)). The total number of analyzed T cell tracks is n=971, n=780, and n=1218 for the non-stimulated, TNF stimulated, and IL1- β stimulated pMBMEC monolayers correspondingly. The data obtained with ^{UFM}Track were in accordance with our previous observations obtained by manual frame-by-frame analysis ([12](#) [14](#) [32](#)). We further obtained the data for T-cell migration speed, T-cell displacement, and T-cell path lengths for the crawling T cells, as these are the primary cell motility parameters. As shown in **Figure 4C-H** [12](#) [14](#) [32](#), we observed statistically significant differences in the mean T-cell motility parameters depending on the pMBMECs stimulation condition. Furthermore, we observed differences in the shape of the distribution of T-cell crawling speeds, consistent with our previous reports of underlying differences in the mechanisms mediating T-cell crawling on non-stimulated versus cytokine-stimulated pMBMECs ([32](#) [12](#) [14](#)). While not statistically significant for the IL1- β and with a low significance for the TNF stimulations, an increased variance of the T cell instantaneous speed on stimulated compared to non-stimulated pMBMECs is observed(**Figure 4G** [12](#) [14](#) [32](#)). This suggests that T-cell crawling is often interrupted by T-cell recognizing specific cues on the stimulated endothelium. The T cell meandering index (MI) distribution was high on non-stimulated and lower on stimulated pMBMECs (**Figure 4H** [12](#) [14](#) [32](#)), underscoring that cytokine stimulation enhances directed T-cell movement on the pMBMEC monolayer.

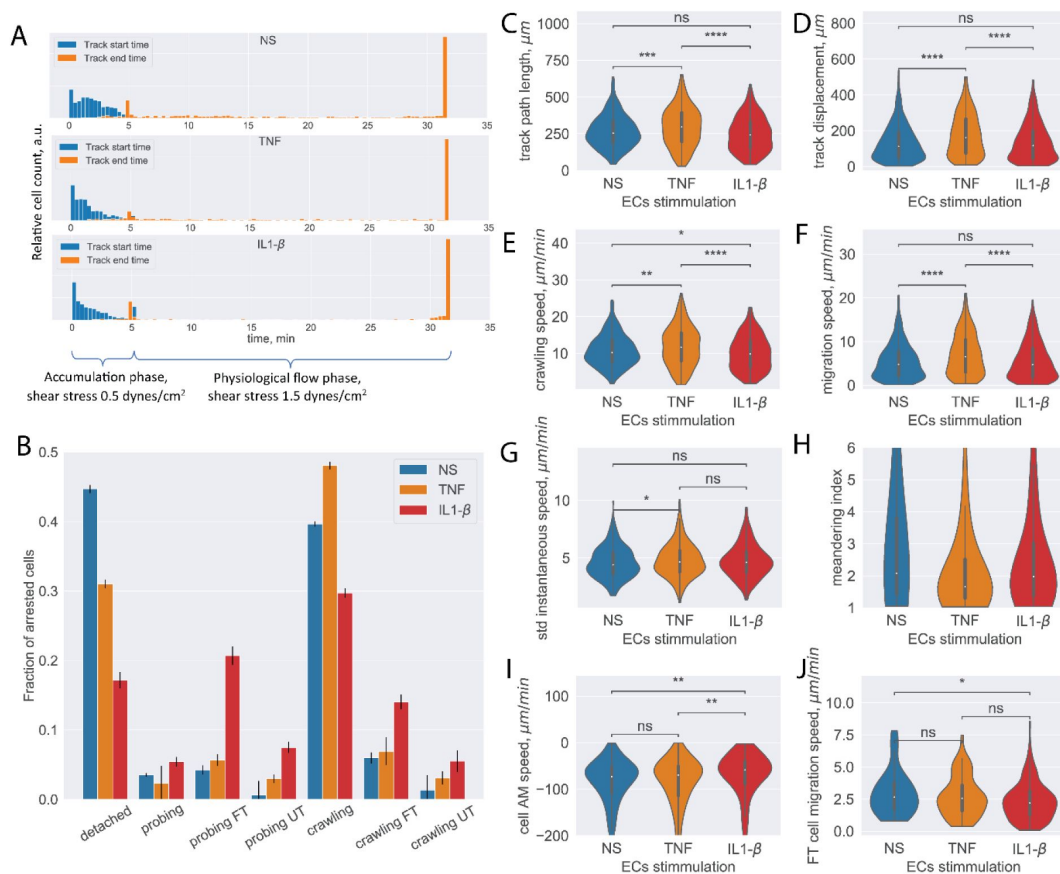


Figure 4.

Analysis of CD4⁺ T-cell tracks. A. Start and end time distribution of CD4⁺ T-cell tracks on non-stimulated or TNF or IL-1 β stimulated pMBMECs. ^{UFM}Track correctly captures increased T-cell detachment from the non-stimulated pMBMECs after 5 minutes when the flow is increased to physiological levels. B. Quantification of CD4⁺ T-cell behavior in the respective categories obtained on non-stimulated and TNF and IL-1 β stimulated pMBMECs. Error bars show the statistical error of the mean. (see text for details). C-H. Motility parameters of the crawling CD4⁺ T cells were obtained for the three endothelial stimulatory conditions. Distributions of T-cell path length (C), displacement (D), crawling speed (path/time) (E), migration speed (displacement/time) (F), variability of instantaneous T-cell crawling speed along the track (standard deviation, G), and meandering index (H). I. Distribution of CD4⁺ T-cell accelerated movement (AM) speed is a proxy metric for the T-cell adhesion to the healthy or inflamed endothelium. J. Migration speed distribution of the transmigrated CD4⁺ T cells. Stimulation applied to the luminal side of pMBMECs affects T-cell migration at the abluminal side of pMBMECs after their transmigration.

FT - T cells performed full transmigration; UT - T cells performed uncompleted transmigration.

Since the analysis was performed automatically with ^{UFM}Track, it allowed for deeper insights into the T-cell migration behavior on the pMBMEC monolayers than obtained by manual frame-by-frame analysis. Specifically, the detection of accelerated movement by ^{UFM}Track enables the researcher to quantify the kinetics of individual T-cell detachment from the endothelium rather than simply quantifying a bulk T-cell detachment rate. This detachment kinetics is reflected in the average speed experienced during accelerated movement, which is significantly lower for the IL1-b condition (**Figure 4I**), suggesting the need to break more bonds with the endothelium when pro-inflammatory cytokines are present.

As our ^{UFM}Track workflow achieves sufficient segmentation efficiency to detect T cells below the pMBMEC monolayer and T-cell transmigration across the pMBMEC monolayer, it also enables investigation of T-cell movement after the transmigration step. In **Figure 4J**, we show the distribution of the migration speed for the transmigrated CD4⁺ T cells. Interestingly, cytokine stimulation of pMBMECs, although applied from the luminal side, also affected T-cell movement at the abluminal side of the pMBMEC monolayer. While in the microfluidic device used in the present study, the migration of T cells below the pMBMEC monolayer may not be biologically relevant, this analysis option will be valuable for future studies involving multilayer in vitro BBB models that include the vascular basement membrane in addition to pericytes and astrocytes to mimic the entire neurovascular unit (8,33).

Importantly, our ^{UFM}Track automatically detects and characterizes unusual events, such as reverse T-cell transmigration, that are easily overlooked with manual counting. We do not present the statistics here, as only a few such events were observed. However, when applied to the multilayer BBBs in forthcoming in vitro models, systematic detection and analysis of these rare events will be important to understand T-cell migration in immune surveillance.

2. CD8⁺ T cells analysis

As we have previously shown that the multi-step T-cell extravasation across pMBMEC monolayers differs between CD4⁺ and CD8⁺ T cells (13), we next analyzed two datasets studying the multi-step migration of CD8⁺ T cells across non-stimulated (NS) and TNF/interferon-gamma (TNF/IFN-γ) stimulated pMBMEC monolayers under physiological flow over 161 timeframes (27 minutes). The CD8⁺ T cells were slightly different in size and appearance when compared to the CD4⁺ T cells used for training the segmentation model. To benchmark our established ^{UFM}Track pipeline in this more difficult configuration, the datasets were first manually analyzed by four experimenters: one advanced experimenter with four years of experience (AdEx) and three inexperienced experimenters who received comparable 2-hour introduction and training (Ex1-Ex3). The analyses were performed on the subset of the acquisition, 161 timeframes long starting from timeframe 31.

Manual cell analysis and tracking were performed as described in the “Materials and Methods” section separately for each of the 8 tiles of the tiled acquisition. Next, all crawling CD8⁺ T cells that did not perform transmigration were manually tracked for the timespan after the flow was increased to the physiological level using the manual tracking in ImageJ.

First, we compared the CD8⁺ T cell behavior statistics obtained manually and by our automated analysis pipeline (**Figure 5A-C**). The results obtained by the automated analysis were in full agreement with the manual analysis performed by the experienced experimenter. At the same time, the data highlight the variability of the results from the inexperienced experimenters, confirming the potential for significant experimenter bias.

We also show the detection efficiency of T cells by the ^{UFM}Track. To achieve this goal, we combined the numbers of CD8⁺ T cells detected in each tile by manual analysis. Due to the overlap between the individual tiles, some CD8⁺ T cells are seen more than once. To obtain an estimate of the total CD8⁺ T cell count detected manually, we scaled the number of CD8⁺ T cells accordingly to

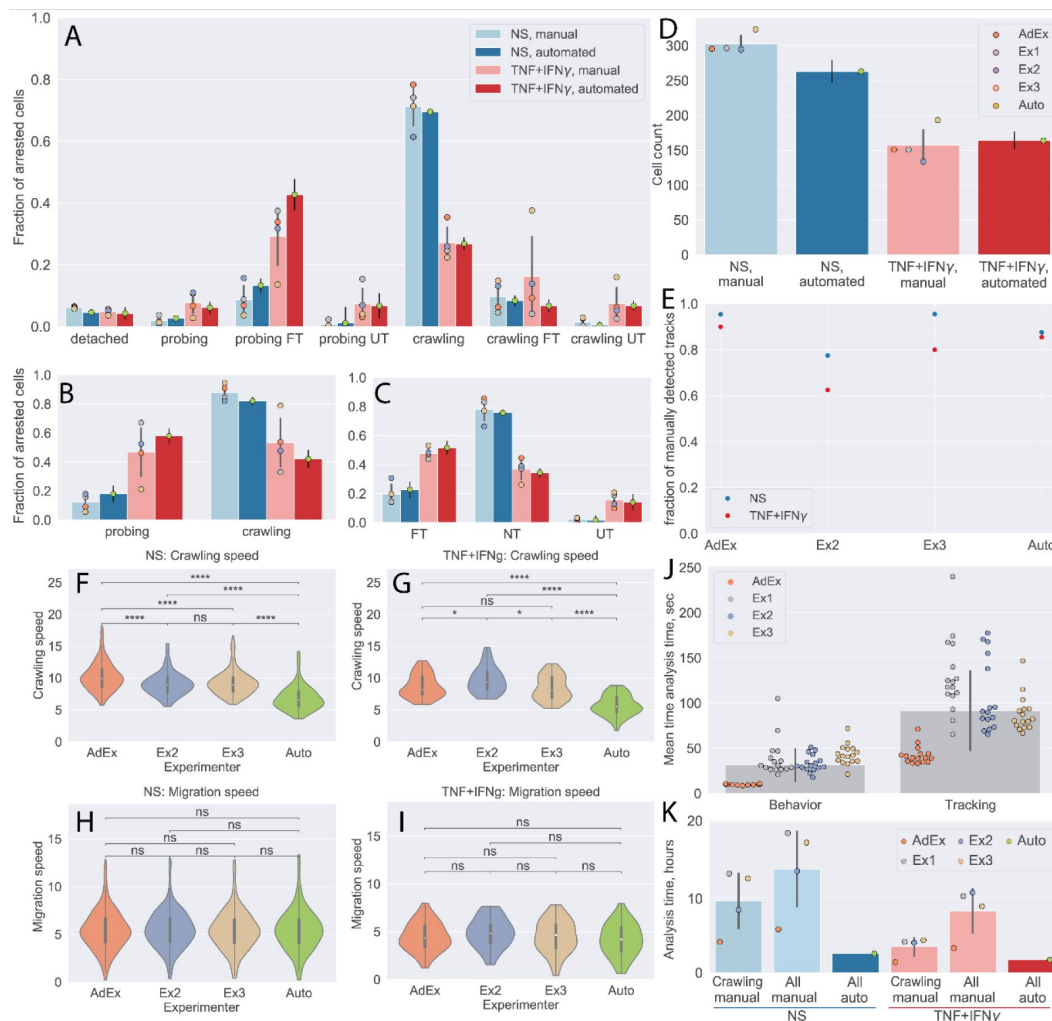


Figure 5.

Comparison of automated analysis with UFMTrack and manual analysis of the CD8⁺ T-cell tracks. A-C: CD8⁺ T-cell behavior statistic obtained for non-stimulated (NS) and cytokine-stimulated pMBMECs as obtained manually by four experimenters, as well as automatically with UFMTrack. A. Quantification of CD8⁺ T-cell behavior in the respective categories obtained on non-stimulated and TNF/IFN- γ stimulated pMBMECs is consistent with results obtained by manual frame-by-frame analysis. Cytokine stimulation of pMBMECs increases T-cell probing behavior (B) and T-cell transmigration rate (C). Error bars show the standard deviation of the manual analysis and the statistical error of the mean for automated analysis. Points correspond to individual experimenters. D. Counts of CD8⁺ T cells were obtained manually by four experimenters and automatically by UFMTrack. The T-cell detection efficiency is above 90%. Error bars show the standard deviation of the manual analysis and the statistical error of the mean for the automated analysis. Points correspond to individual experimenters. E. Detection efficiency of crawling CD8⁺ T cell tracks between the manual and automated analysis. F-I. Comparison of CD8⁺ T-cell crawling speed (path/time) (F, G) and migration speed (displacement/time) (H, I) on non-stimulated (F, H) and cytokine-stimulated (G, I) pMBMECs. The T-cell position assignment error in manual tracking leads to biased crawling speed estimation. J. Comparison of the analysis time (per cell) required for behavior analysis and tracking of CD8⁺ T cells. K. Total analysis time (per dataset) for behavior analysis and tracking of CD8⁺ T cells. Comparison is shown for manual analysis with tracking of crawling cells only (Crawling manual), the time estimate for manual analysis with tracking of all cells (All manual), and the in-depth automated analysis of all cell tracks with UFMTrack (All auto). FT - T cells performed full transmigration; UT - T cells performed uncompleted transmigration; NT - T cells did not perform transmigration.

the number of detected unique T-cell tracks (see next paragraph). By this approach, we found T-cell detection efficiency to be above 90% (**Figure 5D** [↗](#)). The increased T-cell density can explain lower efficiency in the NS condition. These findings set the appropriate T-cell density for automated analysis of their migration behavior on pMBMEC monolayers to 200-300 cells per FoV of the size of 3.8 mm² or 50-80 cells/mm².

Next, we compared the T-cells tracks as obtained manually by three experimenters as well as automatically by our ^{UFM}Track. To this end, we first matched the tracks in adjacent tiles to obtain the tracks on the whole imaging area, avoiding multiple counts of the same track. This was achieved by pattern matching with initial offsets between tiles obtained by an automatic frame alignment procedure performed as part of the automatic analysis. We considered T-cell tracks observed in different tiles to be tracks of the same T cell if $f_{in} > \frac{1}{2}f_{out} - 0.8$, where f_{in} is the fraction of timepoints along a track at which the distance between the T cells was below 17 μm and f_{out} was the fraction of timepoints along a track at which distance between cells was above 25 μm. We used the same approach to match the T-cell tracks obtained by each experimenter and the automated ^{UFM}Track. To compare performance in an objective manner, we excluded manually obtained tracks that lay outside of the fiducial volume of the automated analysis, as well as T cells that were touching another T cell at the end of the acquisition, as those were also excluded from the automated analysis. We then took as 100% the sum of all T-cell tracks detected manually. We evaluated the fraction of all T-cell tracks detected by each experimenter as well as automatically by our ^{UFM}Track.

In **Figure 5E** [↗](#), we show that our pipeline achieves a comparable T-cell tracking efficiency when compared to the manual analysis. While its performance was not superior to that of the experimenter with four years of expertise in the analysis of the under-flow datasets, it does perform better than less experienced experimenters.

We also compared the T-cell motility parameters obtained manually and automatically for the non-stimulated and stimulated pMBMECs (**Figure 5F-I** [↗](#)). Data obtained for the CD8⁺ T-cell migration speed on pMBMECs was comparable between all experimenters and the automated analysis. In contrast, results obtained for the T-cell crawling speed were significantly different between the automated approach and manual analysis. Taking a closer look at the T-cell tracks (Supplementary Figure 6), we readily observed that the manual analysis contains significant errors in the assignment of the T-cell position. This leads to a jittery pattern in the T-cell migration tracks and overestimating the T-cell path and, thus, T-cell crawling speed. Employing our novel automated analysis pipeline eliminated this systematic error in the measurements.

To finally investigate the potential benefit in the time required for automated versus manual data analysis, we examined the time required for a given experimenter to analyze such datasets manually using the Clockify time tracker ([34](#) [↗](#)). In **Figure 5J** [↗](#), we show the average time investment needed to perform a complete T-cell behavior analysis and T-cell tracking in each imaging tile, as well as on average. Clearly, the experienced experimenter outperforms the inexperienced experimenters by a factor of 3. On average, a researcher would thus spend 8.1 hours analyzing a dataset with 300 T cells when only the tracks of crawling T cells (50%, i.e., 150 cells) are analyzed. If all T-cell tracks were to be analyzed, this time would further increase to 12.9 hours. Given that on average 10 datasets can be produced per day, manual analysis becomes a bottleneck, leading to delays in exhaustive data analysis and thus ultimately in research progress. The ^{UFM}Track framework reduces analysis time by a factor of 3 when analyzing only crawling cells and 5 if all cell tracks are to be analyzed (**Figure 5K** [↗](#)). With an average analysis time of 2.3 hours, the 10 experiments carried out in one day can be thoroughly analyzed within one day of machine time. This enables the scalability of flow-based immune cell migration experiments while simultaneously lifting the burden of tedious and time-consuming manual analysis from the researchers.

V. Applicability of ^{UFM}Track to different models of immune cell - endothelial cell interactions

To assess the broader applicability of the ^{UFM}Track framework to different cell types and BBB models employed for the *in vitro* under-flow cell trafficking assays. To this end, we have performed the automated analysis of the multi-step T-cell migration cascade in a set of datasets (one to eight) across different cell types and assays. Specifically, we attempted to verify the feasibility of analysis of datasets where the cells forming the monolayer or the migrating cells have noticeably different appearances, cells from other species are used, specifically human-derived cells, and datasets with a different imaging modality. These include human peripheral blood mononuclear cells (hPBMCs), human T cells, bone marrow-derived macrophages (BMDM) interacting with a monolayer of mouse- and human-derived brain microvascular endothelial cells as well as with immobilized recombinant BBB adhesion molecules (See “Material and Methods” section for details). To assess the performance of the model in each condition, we have performed a visual inspection of the cell masks, transmigration masks, and cell tracks for few datasets in each assay type. We then analyzed cell behavior distribution or motility parameters relevant in each case.

1. hPBMC on HBMEC monolayer

The interaction of hPBMCs with the BBB is altered under different disease conditions. We have therefore analyzed a dataset of human PBMCs on human brain microvascular endothelial cells (HBMEC) to quantify the cell behavior statistic. While the endothelial cells look significantly different than the pMBMECs, the segmentation and transmigration detection quality was sufficient to perform the cell migration analysis. This was also confirmed by the visual inspection of the cell and transmigration masks and cell tracking results (**Figure 6 A** [↗](#)). We then quantified the PBMC behavior distribution and the crawling and migration speed of the crawling PBMC (**Figure 6 B, C** [↗](#)).

2. BMDM on the pMBMEC monolayer

Invasion of macrophages into the CNS contributes to many neurological disorders. Therefore, we have explored if ^{UFM}Track also allows us to analyze the interaction of BMDMs with the BBB under physiological flow *in vitro*. To this end, we have analyzed two datasets of mouse BMDMs interacting with pMBMEC monolayers (**Figure 6 D** [↗](#)). Despite the different behavior and appearance of the macrophages as compared to the T cells, the segmentation quality was sufficient to perform the cell tracking and analyze cell motility parameters (**Figure 6 E, F** [↗](#)), as confirmed by visual inspection of the macrophages masks and cell tracking results (**Figure 6 D** [↗](#)). Nonetheless, due to the different appearance of the flattening crawling cells and the transmigrated cells, the transmigration detection trained on the mouse T cells does not perform sufficiently well for the robust detection of transmigrating macrophages.

3. Human T cells on EECM-BMEC monolayer

Next, we analyzed eight datasets of human T cells interacting with the human induced pluripotent stem cell (hiPSC) derived extended endothelial cell culture method brain microvascular endothelial cell (EECM-BMEC)-like cell monolayers (**Figure 6 G** [↗](#)). EECM-BMECs establish barrier properties comparable to primary human BMECs and display a mature immune phenotype allowing to perform studies for the first time in a fully autologous manner of immune cell interactions with patient-derived *in vitro* models of the BBB ([35](#) [↗](#)). Despite the cells being of different species, the T cells were successfully segmented, and their transmigration was detected, as confirmed by visual inspection of the T-cell and transmigration masks and cell tracking results (**Figure 6 G** [↗](#)).

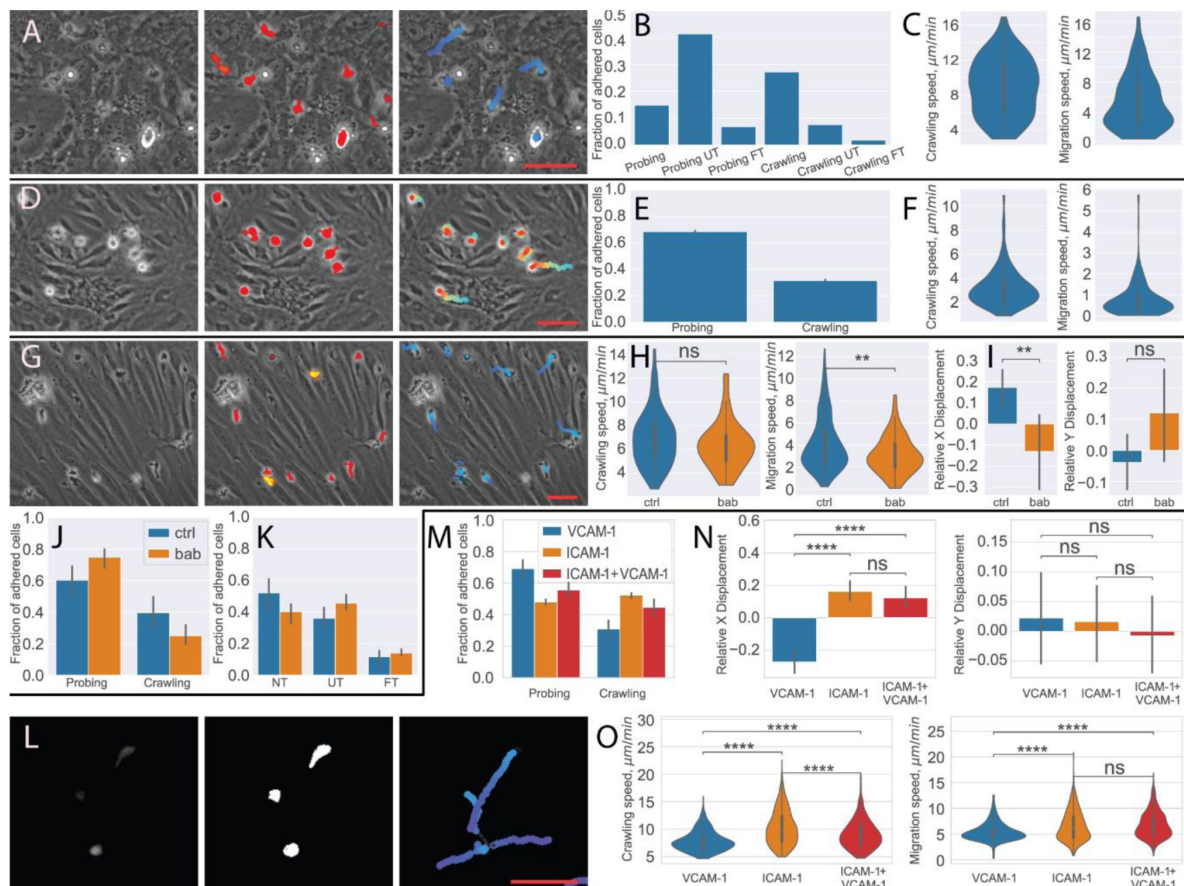


Figure 6.

Analysis of different BBB models with UFMTrack. A-C. Human PBMCs interacting with HBMEC. A. Raw image frame, segmentation, and tracking results. B. PBMCs behavior distribution. C. Crawling and migration speed distribution. D-F. BMDM interacting with the pMBMEC monolayer. D. Raw image frame, segmentation, and tracking results. E. BMDM crawling vs probing behavior distribution. F. Crawling and migration speed distribution. G-K. Human T cells interacting with the EECM BMEC monolayer. G. Raw image frame, segmentation, and tracking results. H. Crawling and migration speed distributions for the control condition (ctrl) and with the blocking anti-bodies (bab). I. X and Y relative cell displacement. J. Crawling vs probing behavior comparison. K. Transmigrations behavior comparison. L-O. Human T cells interacting with immobilized recombinant BBB adhesion molecules. L. Raw fluorescent image frame, thresholding-based segmentation, and tracking results. M. Crawling vs probing behavior distribution. N. X and Y relative displacement. O. Crawling vs probing speed comparison. Scale bar 50 μm . Statistical tests are performed with the Mann-Whitney U test.

We have confirmed the differences in migration statistics (**Figure 6 J, K**), migration and crawling speed of the crawling T cells (**Figure 6 H**), as well as the previously reported difference in relative (normalized to track pathlength) displacement (**Figure 6 I**) of the T cells treated with anti- $\alpha 4$ -integrin + anti- $\beta 2$ -integrin blocking antibodies (bab) and control (ctrl) condition (36).

4. Human T cells on immobilized recombinant BBB adhesion molecules

Finally, we have analyzed datasets acquired in a different imaging modality. Seven datasets of human T cells interacting with immobilized recombinant BBB adhesion molecules, namely ICAM-1, VCAM-1, and their combination (**Figure 6 I**). In this case, the fluorescently labeled cells were imaged with epi-fluorescent microscopy. In the absence of a monolayer, transmigration detection is not necessary. Thus we performed cell segmentation directly on the fluorescent signal (See Materials and Methods for details) using the same segmentation strategy as for the T-cell masks. The tracking was then performed the same way as for other experiments. The T cells were successfully segmented and tracked, as confirmed by visual inspection. We compared T cell behavior and motility parameters for three conditions of the recombinant adhesion molecules: ICAM-1, VCAM-1, and ICAM1 + VCAM1 (**Figure 6 M, O**). Consistent with previous observations (12,36) where the analysis was performed using the ImageJ (26) either manually or with the TrackMate (25) plugin, we observed that in the absence of ICAM-1, T cells were moving in the direction of flow (**Figure 6 N**). At the same time, ^{UFM}Track allows for capturing the rapid accelerated motions of the cells and thus reconstructing longer continuous T cell tracks. This enables capturing the migrations properties of the consecutive steps of the T cell migration cascade on the cell-by-cell basis.

Discussion

In this study, we have developed the under-flow migration tracker (^{UFM}Track) framework. It consists of independent modules and allows for segmentation (**Figure 2**), tracking (**Figure 3**), and motility analysis of T cells, migrating on, across, and below (**Figure 1D**) the monolayer of brain microvascular endothelial cells under physiological flow in vitro. The developed method relies exclusively on phase-contrast imaging data (**Figure 1B**). Therefore, it does not require establishing fluorescent labels of the migrating immune cell population to be studied, avoids potential photo-toxicity to be considered for fluorescent imaging modalities (37–39), and is thus the preferred choice for analyzing the trafficking of sensitive cell types. T-cell segmentation and prediction of the transmigrated T-cell areas are performed using a custom 2D+T U-Net-like convolutional neural network. It enables reliable segmentation of T cells both above and below the pMBMEC monolayer. The existing particle and cell tracking toolkits consider migration of one cell type and thus are not suitable for the detection of distinct migration regimes and cell interactions (23–25,28). Furthermore, to our knowledge, none of the existing algorithms consider migration under the flow causing rapid cell displacement. The tracking of T-cell interactions with the pMBMECs during all migration regimes under physiological flow required designing a new tracking algorithm considering rapid T-cell appearance, disappearance, and displacement in the field of view caused by the flow. We have also developed approaches that resolve track intersections, i.e., identifying track segments corresponding to the same T cell before and after under-segmented track regions, which are inevitable during T-cell migration. By establishing the detection of T-cell crawling, probing, transmigration, and accelerated movement combined with reliable T-cell tracking, we have enabled the in-depth analysis of distinct migration regimes on a cell-by-cell basis. By reducing the dataset analysis time by a factor of 5, ^{UFM}Track allows for performing a thorough analysis of 10 experiments that a researcher can carry out in one day within one day of machine-time. We have demonstrated that the automated analysis performs on par with manual analysis while improving accuracy and eliminating experimenter bias, and enables scalability of flow-based immune cell migration experiments by reducing the analysis cost and lifting the burden of time-consuming manual analysis.

In this work, we have demonstrated the applicability of the developed framework to the analysis of the multi-step extravasation of CD4⁺ and CD8⁺ T cells across non-stimulated or cytokine-stimulated pMBMEC monolayers under physiological flow. We have also demonstrated that the developed framework allows for automated analysis of T-cell behavior statistics and motility parameters of distinct T-cell migration regimes. Results of the automated analysis of CD4⁺ T-cell behavioral statistics performed with ^{UFM}Track are in agreement with previous studies using manual data analysis (**Figure 4B**) (14). The automated analysis of datasets of CD8⁺ T-cell migration has shown comparable performance with manual analysis performed by one experimenter with four years of analysis experience and three less-experienced experimenters. At the same time, the variance of the results obtained manually showed significant experimenter bias that the automated analysis eliminated (**Figure 5F**). Additionally, the automated analysis allowed for in-depth analysis of all T-cell migration categories and precise evaluation of T-cell motility parameters, which was not achieved by manual T-cell tracking, even by the most experienced user.

We have further demonstrated the applicability of the ^{UFM}Track framework to the analysis of different cell types (BMDM, PBMC), species (human-derived T cells, HBMEC, and EECM BMEC) as well as tracking and analysis of cell migration imaged using the fluorescent imaging modality. In all cases, the cells could be successfully segmented and tracked, and the cell migration analysis was performed on a cell-by-cell basis. The main challenge for the system is reliable segmentation of the T cells and the transmigrated T cells. In the case of HBMEC the monolayer has a dotted pattern unlike the rather smooth pMBMEC. This leads to more false positives in the T-cell segmentation at the level of individual frames, which nonetheless could be efficiently filtered at the level of cell tracking. In the case of the BMDMs, since they have significantly different appearances during transmigration from the T cells, reliable transmigration detection was not obtained. This prevents the system from quantifying the transmigration statistics of the BMDMs, yet the cell detection and thus motility parameter evaluation of BMDMs was successful (**Figure 6 D-F**). For all the other analyzed cell types, both the cell segmentation and transmigration detection model trained on the annotated CD4⁺ T cells also produced transmigration maps of sufficient quality for reliable transmigration detection. The framework was able to successfully detect the cells of sizes that differ by almost a factor of two (Supplementary Figure 7). This demonstrates, that simply by discriminating cells by size, it is possible to extend ^{UFM}Track to study interaction of several types of immune cells migrating on top of a cellular monolayer under flow and imaged by phase contrast.

Quantifying the fraction of transmigrated T cells is crucial for studying the molecular mechanisms governing the infiltration of autoaggressive T cells across the BBB into the CNS parenchyma and the immune surveillance. The role of specific endothelial or T-cell adhesion molecules in influencing the dynamic interactions of T cells with pMBMECs can, e.g., be probed by quantifying the ratio of T-cell crawling behavior to T-cell probing (**Figure 6M**) (14,40,41). The analysis procedure established in the ^{UFM}Track framework is flexible and can be easily extended to evaluate further properties of the migrating cells. For example, the number of times a T cell interrupts its crawling regime by switching to short probing behavior on the endothelium can be evaluated, thus providing information on the distribution of “hot spots” on the endothelium. Additionally, experiment scalability and the analysis on a cell-by-cell basis enable the search for distinct populations of T-cells, e.g., according to the distribution of probing to crawling behavioral ratios. The strength of T-cell adhesion to the endothelium can be probed with the developed framework using the measure of T-cell detachment rate and the distribution of the previously overlooked T-cell accelerated movement occurrences and accelerated movement speed. Finally, the possibility of quantifying the motility parameters of the transmigrated T cells enables future studies involving multilayer in vitro BBB models that include the vascular basement membrane in addition to mural cells such as pericytes and astrocytes mimicking the entire neurovascular unit.

While in this work we have focused primarily on the analysis of mouse T cells interacting with pMBMEC under flow or other immune cells interacting with the endothelial monolayer, the methods we present establish a foundation for a broad range of studies involving in vitro under-flow studies of immune cell trafficking. The tracking algorithm and motility analysis developed in the ^{UFM}Track framework are also directly applicable to the analysis of immune cell interactions with recombinant BBB adhesion molecules under flow. In this case, either phase-contrast or epifluorescent imaging can be employed when studying fluorescently labeled immune cells, as demonstrated. The T-cell segmentation based on deep neural networks can be applied to studies of the trafficking of other immune cell subsets on and across the pMBMEC monolayer. We demonstrated the successful application of the ^{UFM}Track to cells with significantly different appearances, such as the HBMEC cellular monolayers. The application to immune cell trafficking across other endothelial monolayers, including those from different species or vascular beds as well as lymphatic endothelial cells where the cell appearance is drastically different from the ones demonstrated here, is possible but requires fine-tuning of the trained segmentation model. While training the models presented here required a large dataset of annotated T-cell masks, and transmigration masks, this can be largely avoided for future development. Future models can be developed more efficiently by leveraging our existing annotated dataset of T-cell migration on the pMBMECs while employing transfer learning approaches in a multitask framework with weak supervision and fluorescent labels as auxiliary learning targets to adapt the model for the segmentation of cells with different appearances. This can be further improved by adopting self-supervised contrastive or masked learning methods, which have demonstrated significant advancements in model pretraining in recent years (42–45). This is the subject of our future studies. The framework could be employed in combination of other methods, e.g. by incorporating fluorescently labeled endothelial adherens junctions one could differentiate the trans- from the paracellular transmigration in the migrating cells. In this case the transmigration locations detected by the ^{UFM}Track framework could be automatically extracted for analysis. One could employ the tracking under flow implemented in the ^{UFM}Track framework for tracking the immune cells migrating on the vascular wall *in vivo* to capture the accelerated movement of cells. By sharing the data and open-source code of the ^{UFM}Track framework, i.e., the training data used for the segmentation model training, trained models, as well as the model architecture, and full under-flow T-cell tracking and migration analysis pipeline, we hope to encourage the community to pursue these developments to advance the field.

One current limitation of the method is the performance reduction of the T-cell tracking when the density of migrating T cells significantly increases (>250 cells/dataset). Thus, the recommended T cell concentration is about 100-150k/ml. However, to avoid non-physiological interactions between migrating T cells and T cell clumping, the T-cell density should be kept at moderate levels anyway. Additionally, analyzing data from 8 stitched FoVs allows for obtaining comparable statistics. The current analysis of the behavioral statistics quantifies the fraction of different T-cell migration regimes with respect to the number of adhered cells instead of the total T-cell count passing through the microfluidic device. While the number of fast-moving T cells during the accumulation phase cannot be directly counted using the imaging modality employed here, it can be estimated indirectly from the imaging data with additional calibration experiments and a dedicated machine learning model. Another limitation is that currently, we do not consider dividing immune cells. While cell division events happen rarely (<1% of T cells) and are not primary events for the study of immune cell interaction with the BBB model, the modular architecture of our framework will facilitate future extension to detect cell division.

By enabling experiment scalability, unbiased analysis with advanced accuracy, and an in-depth analysis of large datasets of T-cell dynamics under flow, the computational and analytical framework presented here contributes to the 3R principle when studying the interaction of cells derived from animal models by reducing the number of animals to be sacrificed. ^{UFM}Track can be employed for fundamental research of the molecular mechanisms governing immune cell trafficking across a range of vascular beds, screening of pharmaceutical treatments, as well as for

personalized medicine based on the evaluation of treatment efficacy on patient-derived T-cell migration behavior on patient-derived endothelial monolayers. One can furthermore envisage that UFM⁺Track can be suitable to study cancer cell metastasis across different endothelial cell monolayers. Eventually, the developed framework can be extended to real-time operation during image acquisition. Combined with transgenic photo-convertible immune cells allowing for the photoconversion of immune cells according to their behavior will allow for subsequent fluorescent cell sorting and scRNA-Seq analysis. Such advanced studies are needed to reveal the role of genetic differences governing T-cell migration regimes.

Materials and Methods

1. pMBMEC cell culture

Primary mouse brain microvascular endothelial cells (pMBMECs) were isolated from 8-12 weeks old C57BL/6J WT mice and cultured exactly as described before ([12](#), [46](#)). Intact monolayers were stimulated or not with 10 ng/mL of recombinant mouse TNF, 20 ng/mL of recombinant mouse IL1- β for, or 5ng/mL recombinant mouse TNF + 100 U/mL recombinant mouse IFN- γ 16-24 hours prior to the assays as previously described ([32](#)).

2. Human brain microvascular endothelial cells

Human brain microvascular endothelial cells (HBMEC) were kindly provided by Prof. Nicholas Schwab (University of Münster, Germany). They were cultured to confluency on speed coating solution coated μ -Dishes (35 mm, low, iBidi) for 2 days as previously described ([32](#)).

3. Extended endothelial culture method— brain microvascular endothelial cell-like cells

The Extended Endothelial Cell Culture Method (EECM) was used to differentiate human induced pluripotent stem cells (hiPSCs) to brain microvascular endothelial cell (BMEC)-like cells were employed as a human in vitro model of the BBB. In brief, hiPSCs from one healthy control (HC) (cell line ID: LNiSi002-B) were established from erythroblasts in the laboratory of Renaud DuPasquier (University of Lausanne, Switzerland). EECM-BMEC-like cells were cultured to confluency on collagen IV and fibronectin-coated μ -Dishes (35 mm, low, iBidi) for 2 days ([36](#)).

4. T cell and hPBMC preparation

Naïve CD4⁺ and CD8⁺ T-cell isolation: Peripheral lymph nodes and spleens from 2D2 and OT-I C57BL/6J mice were harvested, and single-cell suspensions were obtained by homogenization and filtration through a sterile 100 μ m nylon mesh. A second filtration was applied after erythrocyte lysis (0.83% NH₄Cl, Tris-HCl). 2D2 and OT-I cells were isolated respectively with magnetic CD4⁺ and CD8⁺ T cell selection beads (EasySep, STEMCELL Technologies).

In vitro activation of naïve CD8⁺ T cells: OT-I CD8⁺ T cells were activated as described before ([13](#), [41](#)). Activated CD8⁺ T cells were cultured in IL-2-containing media for 3 days post-activation.

In vitro activation of naïve CD4⁺ T cells: 2D2 CD4⁺ T cells were activated as described before ([32](#)). Activated CD4⁺ T cells were cultured in IL-2-containing media for 24 additional hours.

hPBMC were isolated from buffy coats of healthy donors by Ficoll-Paque™ Plus (Cytiva) density gradient and were frozen and stored in a liquid nitrogen tank until use as previously described in ([36](#)).

Human CD4⁺ T cells were isolated by employing a CD4⁺ T cells isolation kit (Miltenyi Biotec kit) following the provider's instructions. Subsequently, effector/memory CD4⁺ T cells were sorted by Fluorescence-Activated Cell Sorting (FACS) into different Th subsets according to their specific surface expression of chemokine receptors (CCR6⁻CXCR3⁺CCR4⁻ for Th1; CCR6⁺CXCR3⁺CCR4⁻ for Th1*; CCR6⁻CXCR3⁻CCR4⁺ for Th2; CCR6⁺CXCR3⁻CCR4⁺ for Th17) and expanded as previously described ([1](#), [33](#), [40](#), [47](#)–[49](#)).

5. BMDM preparation

BMDMs were prepared as described in (Berve et al., manuscript in preparation). In brief, the mouse bone marrow was harvested and washed through a 100 µm nylon mesh. After 7 days of cell culture were plated in a density of $17\text{--}20 \times 10^6$ cells/mL in BMDM medium supplemented with 5ng/mL of recombinant mouse macrophage colony stimulating factor (mCSF, R&D Biosystems, Minneapolis, USA, 416-ML-500) onto non-treated 100mm tissue culture Petri dishes (Greiner Bio-One, St. Gallen, Switzerland) for 7 days at 37°C and 5% CO₂ the cells were harvested by incubation with 0.05% Trypsin.

6. T cell binding assays under physiological flow conditions

For live-cell imaging of T-cell interaction with recombinant BBB cell adhesion molecules under the physiological flow condition, µ-Dishes (35 mm, low, ibidi) were coated with 1.54 µg/mL human recombinant VCAM-1 (Biolegend) and/or 1.14 µg/mL human recombinant ICAM-1 (R&D system) in DPBS for 1 h at 37°C and blocked with 1.5% (v/v) bovine serum albumin (Sigma-Aldrich) in DPBS overnight at 4°C.

CMFDA prelabelled Th cells were incubated with the isotype control antibody (30 µg/mL) for 30 minutes at 37°C (8% CO₂) prior to imaging (exact antibody concentrations are illustrated in the figure legend). The cells were subsequently perfused on top of the pre-coated dishes at a concentration of 10^6 /mL in MAM as previously described ([12](#), [36](#)).

7. In vitro under-flow T-cell migration assay

We studied the multi-step T-cell migration across monolayers of primary mouse brain microvascular endothelial cells (pMBMECs) in a custom made microfluidic device under physiological flow by in vitro live-cell imaging according to the previously established procedure ([14](#), [32](#)). The custom-made flow chamber was described in depth before ([11](#), [46](#)). The flow chamber was placed on top of the pMBMECs, previously cultured in ibidi µ-Dish to confluency, and connected to the flow system filled with migration assay medium (MAM) ([12](#)). During the accumulation phase, T cells at a concentration between 55k/mL and 166k/mL were perfused on top for 5 minutes under low shear stress of 0.1 dynes/cm², allowing them to settle on top of the pMBMEC monolayer. We used lower T-cell concentration compared to previous studies to enable automated T-cell interaction analysis. Afterward, the flow was increased to physiological levels with a flow shear stress of 1.5 dynes/cm² to study post-arrest T-cell behavior on pMBMECs for 27 minutes (**Figure 1A**, B).

8. Data acquisition

The timelapse imaging was performed during both accumulation and physiological flow phases using phase-contrast imaging at a framerate of 6 frames/minute or 12 frames/minute, subsampled to 6 frames/minute and resolution of 0.629 µm/pixel with a 10x objective. In this modality the acquired images are gray scale, and the T cells, especially after the migration across the pMBMEC monolayer, have a similar appearance to the pMBMECs, making the T-cell segmentation task very challenging (**Figure 1C**). The data was acquired in tiles of $870 \times 650 \mu\text{m}^2$ (1389×1041 pixels) with an overlap of 100 µm, leading to a total acquired image area of $3170 \times 1220 \mu\text{m}^2$. For each experiment, the dataset consists of 30 timeframes of T-cell accumulation and 162 timeframes of

dynamic T-cell interactions with the pMBMEC monolayer under physiological flow. The timestep between sequential timeframes for each tile is 10 s. The total acquired area is thus limited by the acquisition speed or by the flow chamber size.

The HBMEC dataset was acquired as previously described (36). In brief, 2 tiles were acquired of total size 3891×1504 pixels with pixel size $0.69 \mu\text{m}$ and framerate 6 frames/minute. The accumulation phase was 4 minutes, and the physiological flow phase was 5 minutes.

The BMDM dataset was acquired similarly to the T cell interacting with the pMBMEC datasets. In brief, one tile was acquired of size of 2048×1504 or 1388×1040 pixels with pixel size 0.69 or $0.629 \mu\text{m}$ and framerate 6 frames/minute. The accumulation phase lasted 6 minutes, and the physiological flow phase was 25 minutes.

The EECM-BMEC dataset was acquired as previously described (36). In brief, 2 tiles were acquired of total size 3892×1504 or 2638×1048 pixels with pixel size 0.69 or $0.629 \mu\text{m}$ and framerate 6 frames/minute.

The dataset of human T cells on immobilized recombinant BBB adhesion molecules was acquired as previously described (36). In brief, 2 tiles were acquired of total size 3891×1504 pixels with pixel size $0.69 \mu\text{m}$ and framerate 6 frames/minute. The accumulation phase was 4 minutes, and the physiological flow phase was 5 minutes. In this dataset, there is only one compartment, thus excluding any transmigration of the T cells. We, therefore, have employed the preprocessed fluorescent images instead of the cell probability map and transmigration map produced by the segmentation neural network. In this case, the centroids are defined as local maxima localized on the preprocessed fluorescent images. The fluorescent images were preprocessed first by performing Gaussian blur filtering in 2D with a standard deviation of 0.75. Next, thresholding was applied to eliminate the background noise, clipping high brightness values (color values with abundance $<0.3\%$ of threshold abundance), scaling to 0-255 8 bit, and saving in a lossless format. Finally, the 2 tiles were stitched together by matching the integrated color distribution in the X direction in the overlapping area.

9. Manual analysis of T-cell migration

Manual cell analysis and tracking were performed according to the previously established procedure using ImageJ software (ImageJ software, National Institute of Health, Bethesda, MD, USA) (13). The number of arrested T cells was thus counted at timeframe 33 of the subset. The behavior of arrested T cells was defined and expressed as fractions of arrested T cells set to 100% as follows:

- T cells that detached during the observation time (“detached”)
- T cells that migrated out of the FoV detached during the observation time (“out of FoV”)
- T cells that continuously crawled on the pMBMEC monolayer (“crawling”)
- T cells that remained in the same location (displacement less than twice the cell size) while actively interacting with the pMBMEC monolayer (“probing”)
- T cells that crossed the pMBMEC monolayer with or without prior crawling (“crawling full transmigration” and “probing full transmigration”). The event of T-cell transmigration across the pMBMECs monolayer became obvious due to the change of appearance of the transmigrated part of the T cells from phase bright (on top of the pMBMECs monolayer) to phase dark.
- T cells that partially crossed the pMBMEC monolayer, then retracted the protrusions and continued to migrate above the monolayer (“crawling uncompleted transmigration” and “probing uncompleted transmigration”).

10. ^{UFM}Track performance evaluation

The inference of T cell masks and transmigration masks was performed on a dual-CPU Intel(R) Xeon(R) CPU E5-2670 v3 @ 2.30GHz, 256GB RAM node equipped with 8 Graphical Processing Units (GPU) NVIDIA GeForce GTX TITAN X / GeForce GTX 1080. Frame alignment and segmentation were performed on an Intel(R) Core(TM) CPU i7-4771 @ 3.50GHz, 32GB RAM workstation with NVIDIA GeForce GTX TITAN GPU. Cell tracking was performed on a dual-CPU Intel(R) Xeon(R) CPU E5-2643 v2 @ 3.50GHz, 256GB RAM workstation.

11. Performance analysis

The statistical tests are performed with the Mann-Whitney U test. Performance of the segmentation models was evaluated based on the pixel classification metrics evaluated using the scikit-learn library ([50](#)). Specifically, the Average Precision is evaluated on the test set as the area under the Precision-Recall curve, as the weighted mean of precisions achieved at each threshold, with the change in recall from the previous threshold used as the weight. The segmentation thresholds for the cell mask and the transmigrated cell mask predictions were selected to maximize the F1 score.

Authors contribution

M.V. implemented the ^{UFM}Track software framework, conducted automated analysis and performance evaluation, and contributed to experimental design and imaging.

M.V. and B.E. wrote the manuscript with contributions from S.S., S.A., and L.M.

M.V., with contributions from S.A., L.M., and S.S., conceptualized the T-cell migration analysis pipeline.

L.M., S.A., and S.S. conducted wet lab experiments and imaging.

S.A., L.M., A.M., and M.V. performed annotation of the datasets for the segmentation model training.

S.A., L.G., A.P., and M.V. performed manual data analysis.

A.A. and B.E. designed the research together with M.V., acquired funding, and coordinated the collaboration.

Acknowledgements

We thank Kristina Berve for providing BMDM datasets for analysis with the ^{UFM}Track. We owe sincere thanks to Dr. James McGrath and Danial Ahmad (Rochester University, NY, USA) for their detailed and insightful feedback on our manuscript. This work was supported by an UniBe ID Grant to MV, BE, and AA and the Microscopy Imaging Center (MIC) of the University of Bern.

Data Availability

The source code for the T-cell segmentation models, data preprocessing, training, performance evaluation, and inference scripts are available on GitHub at <https://github.com/newworldemancer/UFMSegm>. This repository also contains win64 binaries used for the watershed-based segmentation of the predicted T cell probability maps and transmigration probability maps.

The source code for the under-flow T-cell tracking and migration analysis, along with the scripts used for performance evaluation of the framework, is available on GitHub at <https://github.com/newworldemancer/UFMTrack>.

The training phase-contrast data with manual annotations, the reference datasets for histogram normalization, and trained models are available on Zenodo: [<http://doi.org/10.5281/zenodo.7489557>].

The datasets used for evaluation of our framework are available on Zenodo: [<http://doi.org/10.5281/zenodo.7489972>, <http://doi.org/10.5281/zenodo.7489984>, <http://doi.org/10.5281/zenodo.7489996>, <http://doi.org/10.5281/zenodo.7490012>, <http://doi.org/10.5281/zenodo.7490020>, <http://doi.org/10.5281/zenodo.7490029>].

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Editors

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Reviewer #1 (Public review):

Summary:

It is evident that studying leukocyte extravasation in vitro is a challenge. One needs to include physiological flow, culture cells and isolate primary immune cells. Timing is of utmost importance and a reproducible setup essential. Extra challenges are met when extravasation kinetics in different vascular beds is required, e.g., across the blood-brain barrier. In this study, the authors describe a reliable and reproducible method to analyze leukocyte TEM under physiological flow conditions, including this analysis. That the software can also detect reverse TEM is a plus.

Strengths:

It is quite a challenge to get this assay reproducible and stable, in particular as there is flow included. Also for the analysis, there is currently no clear software analysis program, and many labs have their own methods. This paper gives the opportunity to unify the data and results obtained with this assay under label-free conditions. This should eventually lead to more solid and reproducible results.

Also, the comparison between manual and software analysis is appreciated.

Weaknesses:

The authors stress that it can be done in BBB models, but I would argue that it is much more broadly applicable. This is not necessarily a weakness of the study but more an opportunity to strengthen the method. So I would encourage the authors to rewrite some parts and make it more broadly applicable.

<https://doi.org/10.7554/eLife.91150.2.sa3>

Reviewer #2 (Public review):

Summary:

This paper develops an under-flow migration tracker to evaluate all the steps of the extravasation cascade of immune cells across the BBB. The algorithm is useful and has important applications.

Strengths:

The algorithm is almost as accurate as manual tracking and importantly saves time for researchers. The authors have discussed how their tool compares to other tracking methods.

Weaknesses:

Applicability can be questioned because the device used is 2D and physiological biology is in 3D. However, the authors have addressed this point in their revised manuscript.

<https://doi.org/10.7554/eLife.91150.2.sa2>

Reviewer #3 (Public review):

Summary:

The authors aimed to establish a faster and more efficient method of tracking steps of T-cell extravasation across the blood brain barrier. The authors developed a framework to visualize, recognize and track the movement of different immune cells across primary human and mouse brain microvascular endothelial cells without the need for fluorescence-based imaging. The authors succinctly describe the basic requirements for tracking in the introduction followed by an in-depth account of the execution.

Weaknesses and Strengths:

Materials & methods and results

(1) The methods section also lacks details of the microfluidic device that the authors talk about in the paper. Under physiological shear stress, the T-cells detach from the pMBMEC monolayer, and are hence unable to be detected; however, this observation requires an explanation pertaining to the reason of occurrence and potential solutions to circumvent it to ensure physiologically relevant experimental parameters.

(2) The author describes a method for debris exclusion using UFMTrack that eliminates objects of <30 pixels in size from analysis based on a mean pixel size of 400 for T lymphocytes. However, this mean pixel size appears to stem from in-vitro activated CD8 T cells, which rapidly grow and proliferate upon stimulation. In line with this, activated lymphocytes exhibit increased cytoplasmic area, making them appear less dense or "brighter" by phase microscopy compared to naïve lymphocytes, which are relatively

compact and subsequently appear dimmer. Given this, it is not clear whether UFMTrack is sufficiently trained to identify naïve human lymphocytes in circulating blood, nor smaller, murine lymphocytes. Analysis of each lymphocyte subtype in terms of pixel size and intensity would be beneficial to strengthen the claim that UFMTrack can identify each of these populations. Additionally, demonstrating that UFMTrack can correctly characterize the behavior of naïve versus activated lymphocytes isolated from murine and human sources would strengthen the claim that UFMTrack can be broadly applied to study lymphocyte dynamics in diverse models without additional training

(3) Average precision was compared to the analysis of UFMTrack but it is unclear how average precision was calculated. This information should have been included in the methods section

(4) CD4 and CD8 T cells exhibit distinct biology and interaction kinetics driven in part by their MHC molecule affinity and distinct receptor expression profiles. Thus, it is unclear why two distinct mechanisms of endothelial cell activation are needed to see differences between the populations.

(5) The BMECs are barrier tissues but were cultured on μ dishes in this study. To study the transmigration of T-cells across the endothelium, the model would have been more relevant on a semi-permeable membrane instead of a closed surface.

(6) Methods are provided for the isolation and expansion of human effector and memory CD4⁺ T cells. However, there is no mention of specific CD4⁺ T cell populations used for analysis with UFMTrack, nor a clear breakdown of tracking efficiency for each subpopulation. Further, there is no similar method for the isolation of CD8⁺ T cell compartments. A clear breakdown of the performance efficiency of UFMTrack with each cell population investigated in this study would provide greater insight into the software's performance with regard to tracking the behavior and movement of distinct immune populations.

(7) The results section is quite extensive and discusses details of establishment of the framework while highlighting both the pros and cons of the different aspects of the process, for example, the limitation of the two models, 2D and 2D+T were highlighted well. However, the results section includes details which may be more fitting in the methods section.

(8) A few statements in the results section lacked literary support, which was not provided in the discussion either, such as support for increased variance of T-cell instantaneous speed on stimulated vs non-stimulated pMBMECs. Another example is the enhancement of cytokine stimulation directed T-cell movement on the pMBMECs that the authors observed but failed to relay the physiological relevance of it. The authors don't provide enough references for developments in the field prior to their work which form the basis and need for this technology.

(9) The rationale for use of OT-1 and 2D2-derived murine lymphocytes is unclear here. The OT-1 model has been generated to study antigen-specific CD8⁺ T cell responses, while the 2D2 model has been generated to recapitulate CD4 T cell-specific myelin oligodendrocyte glycoprotein (MOG) responses.

Figures and text

(1) There are certain discrepancies and misarrangement of figures and text. For example, discussion of the effect of sheer flow on T cell attachment as part of the introduction in Figure 1 and then mentioning it in the text again in the results section as part of Figure 4 is repetitive.

(2) Section IV, subsection 1 of the results section, refers to 'data acquisition section above' in line 279, however the said section is part of materials and methods which is provided towards the end of the manuscript.

(3) There are figures in the manuscript that have not been referenced in the results section, for example, Figures 3A and B. Figure 1 hasn't been addressed until subsection 7 of materials and methods

(4) A lack of significance but an observed trend of increased variance of T cell instantaneous speed is reported in line 296-298; however, the graph (figure 4G) shows a significant change in instantaneous speed between non-stimulated and TNF α -stimulated systems. This is misleading to the readers.

(5) The authors talk about three beginner experimentors testing the manual T cell tracking process but figure 5 only showcases data from two experimentors without stating the reason for excluding experimentor 1.

Discussion

(1) While the discussion captures the major takeaways from the paper, it lacks relevant supporting references to relate the observation to physiological conditions and applicability.

(2) The discussion lacks connection to the results since the figures were not referenced while discussing an observed trend

(3) The authors briefly looked into mouse and human BMECs and their individual interaction with T-cells, but don't discuss the differences between the two, if any, that challenged their framework.

(4) Even though though the imaging tool relies on difference in appearance for detection, the authors talk about lack of feasibility in detecting transmigration of BMDMs due to their significantly different appearance. The statement lacks a problem solving approach to discuss how and why this was the case.

Relevance to the field:

Utilizing the framework provided by the authors, the application can be adapted and/or utilized for visualizing a range of different cell types, provided they are different in appearance. However, this would require extensive changes to the script and won't be adaptable in its current form.

<https://doi.org/10.7554/eLife.91150.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

It is evident that studying leukocyte extravasation in vitro is a challenge. One needs to include physiological flow, culture cells and isolate primary immune cells. Timing is of utmost Importance and a reproducible setup essential. Extra challenges are met when extravasation kinetics in different vascular beds is required, e.g., across the blood-brain

barrier. In this study, the authors describe a reliable and reproducible method to analyze leukocyte TEM under physiological flow conditions, including this analysis. That the software can also detect reverse TEM is a plus.

Strengths:

It is quite a challenge to get this assay reproducible and stable, in particular as there is flow included. Also for the analysis, there is currently no clear software analysis program, and many labs have their own methods. This paper gives the opportunity to unify the data and results obtained with this assay under label-free conditions. This should eventually lead to more solid and reproducible results.

Also, the comparison between manual and software analysis is appreciated.

We thank the Reviewer for their positive evaluation of our manuscript and highlighting the value of obtaining more reproducible and unbiased results, as well as detection of forward and reverse transmigration with UFMTrack.

Weaknesses:

The authors stress that it can be done in BBB models, but I would argue that it is much more broadly applicable. This is not necessarily a weakness of the study but more an opportunity to strengthen the method. So I would encourage the authors to rewrite some parts and make it more broadly applicable.

We thank the Reviewer for this suggestion. In the revised version of our manuscript, we have now emphasized the broader applicability of UFMTrack to analyze the interaction of immune cells with 2-dimensional endothelial monolayers in various contexts in the abstract, introduction, and discussion sections.

Reviewer #2 (Public Review):

Summary:

This paper develops an under-flow migration tracker to evaluate all the steps of the extravasation cascade of immune cells across the BBB. The algorithm is useful and has important applications.

Strengths:

Algorithm is almost as accurate as manual tracking and importantly saves time for researchers.

We thank the Reviewer for this positive evaluation of our work.

Weaknesses:

Applicability can be questioned because the device used is 2D and physiological biology is in 3D. Comparisons to other automated tools was not performed by the authors.

We thank the Reviewer for pointing our attention to these weaknesses in our manuscript.

We have clarified in the revised manuscript that using 2D endothelial monolayer models in parallel laminar flow chambers is still a state-of-the-art methodology for studying the multi-step extravasation process of immune cells across endothelial monolayers under physiological flow by in vitro live cell imaging. These models provide excellent optical quality that is not yet achieved in 3D models. We have extended the introduction to emphasize the

limitations of existing tools that motivated us to establish UFMTrack. We have furthermore extended the discussion section to highlight the features unique to our UFMTrack framework.

Reviewer #3 (Public Review):

Summary:

The authors aimed to establish a faster and more efficient method of tracking steps of T-cell extravasation across the blood brain barrier. The authors developed a framework to visualize, recognize and track the movement of different immune cells across primary human and mouse brain microvascular endothelial cells without the need for fluorescence-based imaging. The authors succinctly describe the basic requirements for tracking in the introduction followed by an in-depth account of the execution.

We thank the Reviewer for their positive evaluation of our manuscript and highlighting the value of label-free analysis of the multistep immune cell extravasation cascade with UFMTrack.

Weaknesses and Strengths:

Materials & methods and results:

(1) The methods section also lacks details of the microfluidic device that the authors talk about in the paper. Under physiological shear stress, the T-cells detach from the pMBMEC monolayer, and are hence unable to be detected; however, this observation requires an explanation pertaining to the reason of occurrence and potential solutions to circumvent it to ensure physiologically relevant experimental parameters.

We thank the Reviewer for pointing out this oversight. We have used a custom-made microfluidic device that has been published and described in detail before. This information has now been included in the Methods Section under Point 7, and the two references describing the flow chamber in depth are mentioned below and have been included in the manuscript.

Coisne Caroline, Ruth Lyck and Britta Engelhardt. 2013. Live cell imaging techniques to study T cell trafficking across the blood-brain barrier *in vitro* and *in vivo*. *Fluids and Barriers of the CNS* 10:7 doi:10.1186/20458118-10-7; 21 January 2013

Lyck R, Hideaki Nishihara, Sidar Aydin, Sasha Soldati and Britta Engelhardt. 2022. Modeling brain vasculature immune interactions *in vitro*. Angogenesis, 2nd edition. Editors PatriciaD'Amore and Diane Bielenberg *Cold Spring Harb Perspect Med* doi: 10.1101/cshperspect.a041185

T cell detachment is a physiologically relevant parameter besides T cell arrest, polarization, crawling, probing, and transmigration during the interaction with an endothelial monolayer. T cell detachment means that post-arrest, the T cell cannot engage adhesion molecules required for subsequent polarization and, eventually, transmigration.

(2) The author describes a method for debris exclusion using UFMTrack that eliminates objects of <30 pixels in size from analysis based on a mean pixel size of 400 for T lymphocytes. However, this mean pixel size appears to stem from in-vitro activated CD8 T cells, which rapidly grow and proliferate upon stimulation. In line with this, activated lymphocytes exhibit increased cytoplasmic area, making them appear less dense or "brighter" by phase microscopy compared to naïve lymphocytes, which are relatively compact and subsequently appear dimmer. Given this, it is not clear whether UFMTrack is sufficiently trained to identify naïve human lymphocytes in circulating blood, nor

smaller, murine lymphocytes. Analysis of each lymphocyte subtype in terms of pixel size and intensity would be beneficial to strengthen the claim that UFMTrack can identify each of these populations. Additionally, demonstrating that UFMTrack can correctly characterize the behavior of naïve versus activated lymphocytes isolated from murine and human sources would strengthen the claim that UFMTrack can be broadly applied to study lymphocyte dynamics in diverse models without additional training

We thank the Reviewer for the suggestion to more precisely evaluate the range of cell sizes that can be analyzed by our framework. We have included a visualization of crawling cell sizes successfully analyzed by the UFMTrack in Supplementary Figure 7. It demonstrates that the human peripheral blood mononuclear cells, that are almost twice as small as the activated mouse CD4 T cells used in these assays, can be successfully segmented, tracked, and analyzed with the UFMTrack framework. Thus, our UFMTrack framework is suitable for a broad application to differentially sized immune cells during their interaction with the endothelial cell monolayer under flow.

(3) Average precision was compared to the analysis of UFMTrack but it is unclear how average precision was calculated. This information should have been included in the methods section

We thank the Reviewer for pointing our attention to the missing information. We have added a subsection, “Performance Analysis”, to the Materials and Methods section, where we describe the statistical methods and the performance metrics used to evaluate the UFMTrack framework.

(4) CD4 and CD8 T cells exhibit distinct biology and interaction kinetics driven in part by their MHC molecule affinity and distinct receptor expression profiles. Thus, it is unclear why two distinct mechanisms of endothelial cell activation are needed to see differences between the populations.

We thank the Reviewer for pointing out that different cytokine stimulations of endothelial cells were used in the assays used here to test our UFMTrack to analyze CD4 and CD8 T cell interactions with the endothelial monolayer. While the Reviewer is correct that CD4 and CD8 T cells use different mechanism to cross the pMBMEC monolayer as show by us (doi: 10.1002/eji.201546251.) and others and that recognition of cognate antigen on MHC class I on pMBMECs will arrest CD8 T cells and lead to CD8 T-cell mediated apoptosis (doi: 10.1038/s41467-023-38703-2.) the focus of the present study was not on comparing CD4 and CD8 T cell interactions with the pMBMEC monolayer but rather to test suitability of UFMTrack to study the different multi-step transmigration of these T cell subsets across the endothelial monolayer.

(5) The BMECs are barrier tissues but were cultured on μ dishes in this study. To study the transmigration of T-cells across the endothelium, the model would have been more relevant on a semi-permeable membrane instead of a closed surface.

We understand the critique of the Reviewer, but laminar flow chambers with endothelial monolayers still provide a state-of-the-art and established methodology to study immune cell migration across endothelial monolayers by in vitro live cell imaging including endothelial cells forming the blood-brain barrier.

(6) Methods are provided for the isolation and expansion of human effector and memory CD4+ T cells. However, there is no mention of specific CD4+ T cell populations used for analysis with UFMTrack, nor a clear breakdown of tracking efficiency for each subpopulation. Further, there is no similar method for the isolation of CD8+ T cell

compartments. A clear breakdown of the performance efficiency of UFMTrack with each cell population investigated in this study would provide greater insight into the software's performance with regard to tracking the behavior and movement of distinct immune populations.

We thank the Reviewer for this comment. Since a fair performance evaluation requires collecting reliable and consistent manual annotations, in this work we have performed such analysis only for the mouse CD8 T-cell population migrating on the pMBMEC monolayer. We have chosen this as a reference since it is a different cell population than the one the segmentation model was trained on. This provides an insight into how high performance is expected when other immune cell types are studied than the ones used for model development.

(7) The results section is quite extensive and discusses details of establishment of the framework while highlighting both the pros and cons of the different aspects of the process, for example the limitation of the two models, 2D and 2D+T were highlighted well. However, the results section includes details which may be more fitting in the methods section.

We thank the Reviewer for highlighting the extensive work carried out in the development of our UFMTrack framework. We decided to include in the results section only the description of key elements and design decisions taken when developing the framework, such as the need to include a time series of images for successful segmentation of the transmigrated cells. At the same time, the majority of implementational details can be found in the Supplementary Material.

(8) A few statements in the results section lacked literary support, which was not provided in the discussion either, such as support for increased variance of T-cell instantaneous speed on stimulated vs non-stimulated pMBMECs. Another example is the enhancement of cytokine stimulation directed T-cell movement on the pMBMECs that the authors observed but failed to relay the physiological relevance of it. The authors don't provide enough references for developments in the field prior to their work which form the basis and need for this technology.

We thank the Reviewer for this comment and for asking for literature references. However, we cannot provide such references as these are original observations we made by employing the UFMTrack framework. This shows that UFMTrack observes T-cell behaviors that have previously been overlooked. Their physiological relevance will have to be explored in separate studies. We have extended the introduction section to include the details on the existing methods developed in the field, as well as their weaknesses that motivated the development of the UFMTrack framework.

(9) The rationale for use of OT-1 and 2D2-derived murine lymphocytes is unclear here. The OT-1 model has been generated to study antigen-specific CD8+ T cell responses, while the 2D2 model has been generated to recapitulate CD4 T cell-specific myelin oligodendrocyte glycoprotein (MOG) responses.

To establish and test the UFMTrack framework, we have made use of the specific T-cell subsets and endothelial cell models we generally use within our research context. Especially for animal work, this is according to the 3R rules requesting to reduce animal experimentation.

Figures and text:

(1) There are certain discrepancies and misarrangement of figures and text. For example, discussion of the effect of sheer flow on T cell attachment as part of the introduction in figure 1 and then mentioning it in the text again in the results section as part of figure 4 is repetitive.

We thank the Reviewer for pointing our attention to this misarrangement. We have adjusted the label of Figure 4 to emphasize that this effect is correctly captured by the UFMTrack.

(2) Section IV, subsection 1 of the results section, refers to 'data acquisition section above' in line 279, however the said section is part of materials and methods which is provided towards the end of the manuscript.

We thank the Reviewer for pointing our attention to this misarrangement. We have adjusted the text to reflect the correct chapter order.

(3) There are figures in the manuscript that have not been referenced in the results section, for example, figure 3A and B. Figure 1 hasn't been addressed until subsection 7 of materials and methods

We thank the Reviewer for pointing our attention to this misarrangement. We have adjusted the text to refer to all figure panels and the clarification of the cell multiplicity estimation in the supplementary information section. References to Figure 1 were added in the introduction section to illustrate the *in vitro* under flow imaging setup as well as the typical T cell behaviors in such experiments.

(4) A lack of significance but an observed trend of increased variance of T cell instantaneous speed is reported in line 296-298; however, the graph (figure 4G) shows a significant change in instantaneous speed between non-stimulated and TNF α -stimulated systems. This is misleading to the readers.

We thank the Reviewer for pointing our attention to this discrepancy. We have expanded the text to indicate a low statistical significance for the TNF and no significance but just a trend for the IL1-beta conditions.

(5) The authors talk about three beginner experimentors testing the manual T cell tracking process but figure 5 only showcases data from two experimentors without stating the reason for excluding experimentor 1.

We thank the Reviewer for pointing our attention to this ambiguity. While both the migration analysis and the manual cell tracking were performed by all three beginner experimenters, the cell tracking data for the first one was unfortunately lost due to a hardware failure.

Discussion:

(1) While the discussion captures the major takeaways from the paper, it lacks relevant supporting references to relate the observation to physiological conditions and applicability.

This study is not about the physiological relevance of the microfluidic devices and immune cells used but rather about advancing methodology to analyze dynamic immune cell behavior on endothelial monolayers under physiological flow. Therefore, the discussion does not extend to comparing the physiological relevance of the specific *in vitro* models employed in this study.

(2) The discussion lacks connection to the results since the figures were not referenced while discussing an observed trend

We thank the Reviewer for pointing our attention to this misarrangement. We have included the references to the relevant figures as well as supporting references.

(3) The authors briefly looked into mouse and human BMECs and their individual interaction with Tcells, but don't discuss the differences between the two, if any, that challenged their framework.

We thank the Reviewer for pointing our attention to this weakness. We have added to the discussion section clarifications on the challenges of analyzing the T cell interactions with the HBMEC and the BMDM interactions with the pMBMEC monolayer.

(4) Even though though the imaging tool relies on difference in appearance for detection, the authors talk about lack of feasibility in detecting transmigration of BMDMs due to their significantly different appearance. The statement lacks a problem solving approach to discuss how and why this was the case.

We thank the Reviewer for pointing our attention to this weakness and apologize for the misleading explanation of the problem of analyzing the BMDM sample. Since the transmigrated part of the macrophages differs in appearance from a transmigrated part of a T cell, its detection by a Deep Neural Network trained on the T cell data is worse than that for the T cells. At the same time, the detection performance before the transmigration is sufficient for the BMDM migration analysis. The potential approaches to alleviate this are added to the discussion section.

Relevance to the field:

Utilizing the framework provided by the authors, the application can be adapted and/or utilized for visualizing a range of different cell types, provided they are different in appearance. However, this would require extensive changes to the script and won't be adaptable in its current form.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

The authors should announce in the abstract that the software analysis Track is downloadable and free to use for all researchers. They may consider providing some sort of helpdesk, although I realize that that may run into too much time.

As said above, they stress that it can be done in BBB models, but I would argue that it is much more broadly applicable.

We thank the Reviewer for these suggestions. We have emphasized the broader applicability of UFMTrack in the abstract and pointed out the public availability of the code and data.

Can they add an experiment that shows that it also works for neutrophils for example? I understand that on paper yes it should work, but the neutrophils are of course faster etc.

This is an excellent suggestion, but we tested UFMTrack within the current framework of ongoing research, which does not include the investigation of neutrophil transmigration across endothelial monolayers.

Also, the combination of different leukocytes in one TEM assay would really be a step forward. If the software can detect different-sized leukocytes, then this should be possible.

We thank the Reviewer for this suggestion. We have added Supplementary Figure 7, demonstrating the range of cell sizes that were successfully analyzed by the UFMTrack framework throughout our manuscript. We also added a statement to the discussion that according to this data, “simply by discriminating cells by size, it is possible to extend UFMTrack to study the interaction of several types of immune cells migrating on top of a cellular monolayer under flow.”

Extra challenges: can the method also discriminate between paracellular and transcellular migration modes? In particular for T-cells this is known to happen.

We thank the Reviewer for this suggestion. We have added this to the potential applications of UFMTrack in the discussion section. While this differentiation is not feasible relying solely on the phasecontrast imaging data, UFMTrack can simplify this analysis by providing automatically the predictions of the transmigration locations, for analysis of the fluorescent data of the junctional labels.

Reviewer #2 (Recommendations For The Authors):

This paper develops an under-flow migration tracker to evaluate all the steps of the extravasation cascade of immune cells across the BBB. The algorithm is useful and has important applications. There are several points that need to be addressed, particularly about the claims made by the authors.

Please see the comments below for more details:

- *Lines 88-92: Add a citation for the characteristics of the BBB as a barrier*

We have added two references accordingly.

- *Lines 94-95: Can the authors indicate what models were used for these studies and how those compare to their in vitro model? In addition, can the authors say whether T cells were manually tracked in this study to translate results to the clinic and whether the results were successful when translated to the clinic? This may enhance the argument that automatic trackers are needed if the translation was not 100% successful*

This introductory paragraph summarizes in vivo and in vitro observations from several laboratories. Although these studies include manual tracking of T cells, they do not necessarily distinguish all sequential steps of the multi-step T cell transmigration cascade. Thus, automated tracking may provide additional insights, allowing for increased translation of findings to the clinic.

- *Lines 96-98: Citing the work of Roger Kamm and Noo Li Jeon would be helpful here as they pioneered these BBB microfluidic models and have protocol papers on how to build them and how to use them for cancer cell extravasation studies. Roger Kamm has also worked on several extravasation studies with neutrophils, monocytes, and PBMCs from 3D vasculatures in microfluidic devices, under flow using pressurized fluid or recirculating pumps. Mentioning those would be helpful as they are directly related to what the authors are presenting in their paper.*

We thank the Reviewer for this comment, and we consider the work of Roger Kamm and Noo Li Jeon as very valuable for the field. However, these authors have focused on developing functional 3D microfluidic devices, including, e.g., all cells of the neurovascular unit which is not the focus of this present study that solely employed parallel flow chamber devices and endothelial monolayers.

- *Lines 110-116: Can the authors comment on the use of ImageJ or similar automatic tracking tools and how these compare to the under-flow migration tracker developed in this paper? Several groups use ImageJ to track cellular migration successfully and in an automatic manner with short intervals between each frame. One paper that comes to mind is Chen et al: DOI: 10.1073/pnas.1715932115 where neutrophil migration in 3D was assessed with ImageJ in microfluidic devices of the vasculature. If the authors can highlight differences between their tool and what is currently available and used for automatic tracking (e.g. ImageJ), this would help in understanding the advantages of the migration tracker developed in this paper.*
- *Lines 118-121: Add citations for the current state of the art for T cell extravasation tracking*

We thank the Reviewer for these suggestions. We have extended the introduction to add more details on the available tools for tracking migrating immune cells and their limitations, as well as the discussion section to emphasize the features unique to the developed UFMTrack framework.

- *Figure 1: The device used by the authors is considered to be a 2D microfluidic device with a monolayer of mouse brain endothelial cells. I would recommend the authors to carefully revise the claims made in the paper to mention that this is a 2D device as opposed to a 3D device, in order to not mislead readers who may be expecting these analyses to be performed in 3D vasculatures.*

We thank the Reviewer for this suggestion. We have included in the summary the mention of the 2dimensional nature of the employed BBB model.

- *Figure 1: The T cells used in this study are not fluorescently-labeled but the authors mention that this is an issue from current state-of-the-art tools. I would recommend that the authors remove this point as being an issue because it is not addressed in their paper. The T cells are also not labeled in this study so this limitation of other systems is not addressed in this paper.*

We apologize to the Reviewer as we do not understand this question. There will be many experimental conditions not allowing to study fluorescently tagged T cells. Therefore, UFMTrack is tailored to follow and analyze T cells and other immune cells during their interaction with endothelial monolayers independent of a fluorescence tag.

- *Figure 1: Was the shear stress controlled manually with a syringe? Or with the use of a pressure controller? I would clarify this aspect and discuss human errors that can be introduced from manually controlling the pressure applied to the monolayer.*

We thank the Reviewer for pointing our attention to this ambiguity. We have added a mention of the automated syringe pump used to control the shear stress in the text where the values of shear stress applied to the sample are first mentioned.

- *Figure 1: Does T cell attachment occur within the first 5 minutes? Can the authors comment on how they chose this timeline and the percentage of T cells that are washed*

off at the second step at 1.5 dynes/cm²? Is 30 seconds enough to ensure all the non-adhered T cells are washed off with 1.5 dynes/cm²?

Superfusion of the T cells over the endothelial monolayer is performed under 0.5 dynes/cm² to allow the T cells to settle on the endothelial cell monolayer under flow. After increasing to physiological, flow non adherent T cells detach within 30 seconds, as described by the Reviewer. We have included in the Methods Section Point 7 the references describing in depth the design of the flow chamber device and methods used here.

• Line 154: How many images were used in the training vs. testing dataset for T cell migrations?

We thank the Reviewer for pointing our attention to this missing information. We have added the sizes of the training and validation datasets. Specifically, the 226MPix of available imaging data was split into 154Mpix training and 37 MPix validation sets. The gap in between was introduced to avoid a correlation between validation and training set that would compromise the performance evaluation.

• Are the supplementary videos at real speed or accelerated?

We thank the Reviewer for pointing our attention to this missing information. The videos are sped up by a factor of 96. We have added this information to the Supplementary video descriptions.

• Lines 208-216: Can the authors comment on how their initial adhesion timeframe of 30sec before starting the recording at 5.5min affects the number of T cells with rapid displacement? 30 seconds may not be enough to ensure T cells have adhered to the endothelium

Please see our comment above. The methodology used in the present assays has been set up and validated in numerous publications. We have included in the Methods Section under Point 7 the references describing in depth the design of the flow chamber device and the methods used here.

• Lines 275-277: Was the number of testing images 18? Can the authors comment on how this compares to training dataset size and whether these numbers are enough to achieve robust results?

We apologize for this ambiguity in our manuscript. The framework was evaluated on 18 imaging datasets, each corresponding to 32 minutes of recording, not 18 images. We have added this clarification to the “CD4+ T cell analysis” subsection. The total size of these datasets is 18 datasets * 191 timeframe/dataset * 9.9MPix/frame = 34MPix

• Figure 4B: Can the authors add statistics here? Individual datapoints on the error bars would be helpful too.

We thank the Reviewer for pointing our attention to this weakness. The data corresponds to the statistical errors as evaluated based on all cells in the 18 datasets. We have added the total number of cells in each of the endothelium stimulation conditions to the text.

• Figure 4C-J: Can the authors put individual datapoints here as well and explain whether they considered each T cell to be one datapoint or each endothelium (averaging all T cells) to be one datapoint?

We thank the Reviewer for this suggestion. However, adding about one thousand points corresponding to each cell would be impractical. We thus present the distributions of the evaluated from the data metrics as a histogram on the violin plot instead of the swarm plot.

• *Figure 4: Did the authors wash the monolayers before introducing T cells? Soluble unbound cytokines may still be present and there are two different questions that would be studied here: “Is the inflamed endothelium affecting T cell migration?” (if washing was performed) or “Is T cell and microenvironmental inflammation affecting T cell migration?” (if no washing was performed)*

The endothelial monolayers are “washed” by starting the flow in the flow chamber device and this is before superfusing the T cells over the endothelial monolayer. We agree that our flow chamber device combined with UFMTrack will allow to address all these questions.

• *Figure 4I: Are all the T cells decelerating? (negative AM speed)*

We thank the Reviewer for this question. The cells are moving along the flow, which, in our experiments, is from left to right. The vector of speed is thus pointing against the x-axis, and thus the AM speed is negative.

• *Lines 302-306: Please explain how this compares to ImageJ or similar trackers that can achieve similar outputs.*

We thank the Reviewer for this question. We have added a statement in the “T-cell tracking” section emphasizing that standard trackers are incapable of correctly capturing large displacements.

• *Lines 306-309: It is not lower for TNF stimulation though. How do the authors address this? TNF is also a pro-inflammatory cytokine.*

We have previously shown that stimulation of pMBMECs with IL-1 and TNF- α induces different cell surface levels of ICAM-1 and VCAM-1, which will influence T cell behavior on the pMBMEC monolayer.

• *Lines 313-315: Could this be because the monolayer was not washed and soluble cytokines affected T cell response directly?*

Please see our answer to lines 306-309.

• *Lines 319: Please cite Roger Kamm and Noo Li Jeon’s papers on BBB models with human BMECs, pericytes and astrocytes in 3D microfluidic devices.*

We thank the Reviewer again for pointing out these studies. As mentioned above, as our present study does not explore 3D models of the BBB, we think it does not fit into the framework of our study to elaborate on 3D models of the BBB. In addition, this would require the inclusion of a discussion of the work of others like, e.g., Peter Searson and others.

• *Figure 5: Several statistics are missing from parts of the figure. Please add those.*

We apologize – but we do not understand which statistical analysis the Reviewer is missing from this Figure.

• *Can the authors comment on the number of T cells perfused over the monolayer and if this ratio of T cells to endothelial cells makes physiological sense? Too many T cells may result in endothelium inflammation and increased diapedesis.*

The number of T cells used to supprerfuse over the endothelial monolayer is tested to avoid aggregation of T cells in suspension and thus artificial interactions with the endothelial monolayer. T cell behavior on the pMBMEC monolayer remains the same over the dilution of factor 10.

• *Lines 381 383: How does this compare to analyses that look at the cross-section of the endothelium? It is difficult to assess transmigration looking at the top view of the endothelium. Perhaps, cross-section assessments will identify differences in manual vs. automatic tracking.*

There is, to the best of our knowledge, no microscopic device that would allow for in vitro live cell imaging of a live endothelial monolayer – this is in the presence of tissue culture medium – from the side at a resolution that would allow to define transmigration. Our current study rather shows the UFMTrack can distinguish cells moving above or below the endothelial monolayer.

• *Figure 5J: This is probably the most important argument of the paper. If the authors can show statistical differences in their graph, this would greatly help convince readers that this tool is necessary and actually computationally efficient compared to manual work by researchers.*

We thank the Reviewer for this suggestion. However, comparing a single data point for automated measurement with four manual experimenter analysts is not a statistically sound comparison. We believe that Figure 5K is clearly showing the factor 5 difference in analysis speed as compared to manual analysis. More importantly, though, the automated analysis is taking the machine time, lifting the need for the experimenter to invest even 1/5th of the original analysis time.

• *Figure 6: Did the authors use autologous immune cells and endothelial cells? This is particularly relevant with the use of human-derived T cells (line 436) on the BMEC monolayer. Can the authors comment on non-self reactivity by the T cells encountering BMEC from another human subject?*

Autologous T cell interaction with BMECs would only be possible when using hiPSC-derived EECM-BMECs and the T cells from the same individual. All other experimental frameworks will not include autologous interactions. This is the experimental framework used by most authors studying immune cell interactions with commercially available donors. We have not studied alloreactive interactions in our assays and thus cannot further comment.

• *Figure 6M,N,O: How does this compare to ImageJ for tracking of fluorescent cells? I recommend the authors to try that, at least for this section, as this may enhance their argument for their tool vs. standard tools like ImageJ if success rates are higher for their tool.*

We thank the Reviewer for this suggestion. We included a note on the analysis of the fluorescent datasets using the TrackMate plugin for imageJ performed previously in our lab in the “Human T cells on immobilized recombinant BBB adhesion molecules” subsection.

- *Figure 6: Please put individual datapoints on the bar or violin plots where they are missing.*

We thank the Reviewer for this suggestion. However, adding about one thousand points corresponding to each cell would be impractical. We thus present the distributions of the evaluated from the data metrics as a histogram on the violin plot instead of the swarm plot.

- *Lines 467-471: This argument is important and should be mentioned earlier in the introduction.*

Another point that can be mentioned is the application of this platform to imaging modalities in vivo (mouse or human) given that there is no fluorescent staining in these cases. This review may be relevant: <https://doi.org/10.1002/jcb.10454>

We thank the Reviewer for this suggestion. We have clarified in the introduction that UFMTrack does not require fluorescent labels of the imaged migrating cells and relies solely on the phase contrast imaging data.

- *Discussion: Please address a few more potential applications to this study. One can be cancer and immune infiltration.*

We thank the Reviewer for this suggestion. We have elaborated on additional potential applications to the discussion section.

Reviewer #3 (Recommendations For The Authors):

- (1) *Line 327-328: The authors talk about 'As we have previously shown...pMBMEC monolayers differs between CD4+ and CD8+ cells...'. Where was this shown? If it was in a previously published article, please provide a reference.*

We have added these missing references.

- (2) *Line 353: Please provide clear location on where to find the associated information instead of stating 'see below'.*

We thank the Reviewer for pointing our attention to this ambiguity. We have corrected the phrase to “see next paragraph”

- (3) *Line 439: Please correct the acronym to BMECs*

We thank the Reviewer for pointing our attention to this typo. We have corrected it.

<https://doi.org/10.7554/eLife.91150.2.sa0>