Analysis of signalling mechanisms regulating microglial process movement *in situ*

Running Head: Analysis of microglial dynamics *in situ*

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Abstract

Microglia, the brain's innate immune cells, are extremely motile cells, continuously surveying the CNS to serve homeostatic functions and to respond to pathological events. In the healthy brain, microglia exhibit a small cell body with long, branched and highly motile processes, which constantly extend and retract, effectively 'patrolling' the brain parenchyma. Over the last decade, methodological advances in microscopy and the availability of genetically encoded reporter mice have allowed us to probe microglial physiology *in situ*. Beyond their classical immunological roles, unexpected functions of microglia have been revealed, both in the developing and the adult brain: microglia regulate the generation of newborn neurons, control the formation and elimination of synapses, and modulate neuronal activity. Many of these newly ascribed functions depend directly on microglial process movement. Thus, elucidating the mechanisms underlying microglial motility is of great importance to understand their role in brain physiology and pathophysiology. Two-photon imaging of fluorescently labelled microglia, either *in vivo* or *ex vivo* in acute brain slices, has emerged as an indispensable tool for investigating microglial movements and their functional consequences. This chapter aims to provide a detailed description of the experimental data acquisition and analysis needed to address these questions, with a special focus on key dynamic and morphological metrics such as surveillance, directed motility and ramification.

Keywords: Microglia, surveillance, ramification, directed motility, two-photon imaging, image processing.

1. Introduction

About a decade ago, two seminal studies documented for the first time the highly dynamic nature of microglial processes, using *in vivo* two-photon imaging of genetically labelled microglia [1, 2]. This has greatly changed our view of microglia as merely immune-competent and rather stationary cells, and revealed profound morphological differences when compared to their *in vitro* counterparts [3]. In combination with complementary techniques such as immunohistochemistry, transcriptomics or electrophysiology, two-photon imaging of genetically- or dye-labelled microglial cells has been established as an indispensable tool to elucidate microglial functions in the brain. In this chapter, we describe methodology for studying real-time changes of microglial morphology (ramification) and process dynamics (surveillance and directed motility) *in situ*, by which we mean either *in vivo* or *ex vivo* in acute brain slices. While imaging of microglial process dynamics in *ex vivo* preparations, such as acute brain slices, facilitates pharmacological manipulations to investigate the mechanisms regulating microglial motility, *in vivo* imaging is ideally suited to study the intrinsic cell dynamics in wild-type or genetically modified animals with least tissue disturbance, and is necessary to validate key pharmacological results obtained *ex vivo*.

Here, after outlining the brain slicing procedure, we describe how to image real-time movements of microglial cells by generating 4D two-photon image stacks (x, y, z, t). We then provide detailed instructions on how to process these data for subsequent quantification of microglial surveillance, directed motility and ramification. This paper focuses exclusively on imaging and analysing microglia in acute brain slices, however, most of the equipment and imaging methods detailed here can be readily used *in vivo*. Links are provided to software that can be used to analyse microglial process motility.

2. Materials

2.1 Equipment

1. Two-photon microscope equipped with an infrared femtosecond pulsed laser and data acquisition software. We use LSM710 and LSM780 microscopes (Zeiss, Jena) equipped with Mai Tai DeepSee eHP Ti:sapphire infrared lasers (Spectra-Physics, Santa Clara CA) and the Zen data acquisition software (Zeiss).
2. Water immersion objective lens with high magnification and low numerical aperture (e.g., Zeiss Achroplan 20x NA 1.0) and low-magnification air objective (e.g. 4x or 10x) to facilitate navigation to the desired brain region, or to place glass pipettes for local drug application.
3. Custom-made, mobile, perfusion chamber fixed on the microscope stage and connected to a perfusion inflow and suction.
4. Perfusion system, driven by gravity or a peristaltic pump. Aim for a perfusion rate of 3-5 ml/min to support slice quality.
5. Thermostat set to maintain a temperature of 34-36^{o}C for the perfusate in the slice chamber. Avoid temperature changes (as this will cause slice movements in the z-axis) and especially temperatures close to, or above, 37^{o}C.
6. Harp: Create a U-shaped, flattened, platinum frame (approximately 1-1.5 cm in size) with tightened parallel nylon strings glued across it to securely fix the slice in the perfusion chamber.
7. Softwares for data analysis. We use ImageJ or Fiji (NIH) as free image processing softwares to preprocess 4D (x, y, z, t) image stacks and generate binarized (i.e. digitized) movies (x, y, t) of individual microglial cells. Digitized images are then quantified for motility and ramification using algorithms implemented in programming languages such

as MATLAB (The Mathworks, Natick MA), but free software alternatives can also be used (e.g. Python; www.python.org). Our programmes are available at https://github.com/AttwellLab/Microglia.

2.2 Reagents

1. Animals: ideally use transgenic mice with genetically encoded microglial cells expressing a bright fluorophore such as EGFP or EYFP under a microglial-specific promoter, e.g. Cx3Cr1, Csf1r or Iba-1 (see overview in [4]). Alternatively, if no transgenic mouse line is available, acutely label microglial cells in mouse or rat brain slices using fluorescent dye-coupled lectins [5, 6].
2. The following lectins are suited for acute labeling of microglial cells (both in slices and *in vivo*):
   - Isolectin GS-IB_4 from *Griffonia simplicifolia*, conjugated to Alexa^{TM} Fluor 488 or 594;
   - DyLight (488 or 594) Labeled *Lycopersium esculentum* Lectin.

   Make a stock solution (0.5 mg/ml) in distilled H_2O, prepare aliquots of 50 μl size (containing 25 μg of lectin) and store at -20^{o}C. Stocks can be used for at least 12 months (*see* **Note 1**).

2.3 Solutions

1. **Slicing Solutions:** Use standard slicing solution S1 for young animals (<1-2 month) or adopt a more protective slicing procedure beyond that age (see below), using two different solutions: SP1 for slicing and SP2 for recovery of the slices. Each round of slicing requires ~500 ml of solution S1, or ~250 ml of solutions SP1 and SP2 each, considering the typical volumes of a slicing chamber and slice storage container. The solutions are as follows:

- Standard slicing solution S1: (mM) 124 NaCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1 kynurenic acid, 10 glucose, bubbled with 95%O_2/5%CO_2 and cooled to < 4 ^oC.
- Protective slicing solution SP1: (mM) 93 N-methyl-D-glucamine (NMDG), 93 HCl, 20 HEPES, 30 $NaHCO_3$, 2.5 KCl, 0.5 $CaCl_2$, 10 $MgCl_2$, 1.2 NaH_2PO_4, 25 glucose, 1 kynurenic acid, 5 Na-ascorbate, 3 Na-pyruvate, pH adjusted to 7.4 with HCl, bubbled with 95%O_2/5%CO_2 and cooled to < 4 ^oC.
- Protective recovery solution SP2: (mM) 92 NaCl, 20 HEPES, 30 $NaHCO_3$, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 25 glucose, 1 kynurenic acid, 5 Na-ascorbate, 3 Na-pyruvate, pH adjusted to 7.4 with NaOH, bubbled with 95%O_2/5%CO_2 and warmed to ~33-35^oC (e.g. using a water bath).

2. **Extracellular solutions:** Prepare extracellular solution E1 or E2 to superfuse brain slices at 34-36^oC for *in situ* imaging experiments. The main difference between solutions E1 and E2 is the use of bicarbonate vs. HEPES as a pH buffer. The solution are as follows:

   - Extracellular solution E1: (mM) 140 NaCl, 2.5 KCl, 10 HEPES, 1 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, pH set to 7.4 with NaOH, bubbled with 100% O_2.

   Alternatively, use a bicarbonate instead of HEPES-buffered solution:

   - Extracellular solution E2: (mM) 124 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, bubbled with 95%O_2/5%CO_2 (*see* **Note 2**).

3. Methods

3.1 Acute labelling of microglia with fluorescently tagged lectin dyes

Prepare acute brain slices of your region of interest. Note that microglial density can vary across different brain regions. Regions with a particularly high microglia density include e.g. the hippocampus, basal ganglia or substantia nigra [7, 8]. Consider the age of the animal

when choosing the standard, or the more protective slicing solutions (see 2.3.1 above). In our experience, the standard slicing solution works well for animals up to 1-2 months of age, and may also be fine for older animals. However, to ensure high slice quality beyond that age, we recommend using the more protective solutions (SP1 and SP2), which are designed to minimize slicing-evoked tissue swelling and oxidative stress. A detailed comparison of various slicing solutions and their benefits, especially when using older animals, can be found in [9] and at https://www.brainslicemethods.com.

1. Incubate slices in darkness for 30-45mins, not later than 4hrs after slicing (see **Note 3**), in oxygenated extracellular solution E1 or E2 containing 25 μg/ml fluorescently conjugated tomato- or isolectin B_4 at room temperature (*see* **Notes 1 & 4**).
2. It is recommended to use the wells of a standard 24-well culture plate with an incubation volume of ~1.5 ml (reducing the volume is advisable as lectin dyes are costly). Free-floating slices are gently laid on a nylon net placed ~1-2 mm above the bottom of the well (custom-built, e.g. by spanning a nylon net across an adequately sized plastic ring glued to the bottom of the well) to ensure proper penetration of the lectin to both sides of the tissue (*see* **Note 5**).
3. To maintain oxygenation (and pH, for bicarbonate buffered solutions), gently blow 100% O_2 above the incubation well when incubating in HEPES-buffered solution E1, or 95%O_2/5%CO_2 for bicarbonate-buffered solution E2 (alternatively place the incubation chamber in an incubator pre-set to 5%CO_2).

3.2 Two-photon Imaging of microglial dynamics

The study of the signalling mechanisms underlying microglial dynamics in rodents requires time-lapse imaging of their motility and behaviour, as well as the use of drugs for

pharmacological investigations. Two-photon microscopy of fluorescently labelled microglia can be performed in the intact brain of transgenic mice as well as in acute brain slices of mice and rats. The use of acute brain slices is beneficial if the focus of the study is to elucidate mechanisms, and thus requires accessibility (and washout) of drugs at specific concentrations. On the other hand, *in vivo* brain imaging in the intact brain (thin-skull or open window preparations) is the least invasive method for studying microglial dynamics and, as such, is indispensable for the validation of any results obtained with slices.

Here, we describe the methodology for performing two-photon imaging to assess microglial dynamics in acute brain slices, focusing on technical specifications required for imaging healthy and highly ramified microglia *ex vivo*. Identical data acquisition parameters can be used for *in vivo* two-photon imaging and analysis of microglial dynamics, however, the set-up required for thin-skull or open window preparations is different and has been described previously [10].

1. Oxygenate appropriately the extracellular solution (E1 or E2) at room temperature for at least 30 min before imaging and make sure to maintain sufficient oxygenation throughout the imaging session.
2. Switch on the perfusion system and stabilize the flow at a constant rate of ~3 ml/min (*see* **Note 6**).
3. Stabilise the bath temperature at 34-36^{o}C.
4. Switch on the laser and tune it to the desired wavelength (~900-920 nm for eGFP excitation; ~ 800 nm for isolectin B_4 – Alexa 594 excitation) (*see* **Note 7)**.
5. Transfer a slice with fluorescently labelled microglia (*see* **Note 8)** into the bath under the microscope and stabilise it by gently placing a harp on the top (*see* **Note 9)**.

6. Focus on the hippocampal CA2 area with a low-magnification objective (4x or 10x). Find the desired area and immediately change objective lens to higher magnification (20x) (*see* **Note 10)**. Make sure to use as little illumination as possible so as not to photodamage the tissue during this procedure.
7. Make sure that the two-photon microscope is enclosed in a container or "cage" that can be made absolutely dark (switch off lights and close the curtain or door to the enclosing container) and start imaging (*see* **Note 11)**.
8. Use minimal laser intensity to focus on an area of interest at a depth of ~100 μm into the slice (*see* **Note 12)**. Once you focus on the desired area (Fig. 1), increase the laser intensity to values appropriate for imaging (*see* **Note 7)** and take a stack of typically 20-30 slices at 2μm depth intervals. Recommended image acquisition parameters are: 512x512 pixels, 2 frame averages, 1 μsec pixel dwell time (see **Note 13**).
9. For imaging of baseline microglial surveillance (in the absence of damage or applied drugs), stacks of 21-31 slices at 2 μm depth intervals are acquired every 60 s for ~15-20 min. At the end of the baseline imaging session, observe the maximum intensity projection of the time-lapse recording for possible motion artifacts, microglial activation and/or photobleaching (*see* **Note 14)**. Do not proceed with the application of drugs if microglia processes appear to retract, or if significant motion of the tissue is present. If that happens, remove the slice from the bath, transfer a fresh one to be under the microscope and repeat the imaging procedure as described so far (steps 5-9), until the data you acquire passes this first quality control.
10. For mechanistic studies, apply drugs to the brain slices through the perfusion system and record another time-lapse imaging session using the same acquisition parameters as for the baseline (*see* **step 9**). It is recommended to stop the imaging in between the baseline recording and the application of the drug in order to perform a quality test (*see* **Note 14),**

and possibly to realign the imaged area on the initial stack in case of drift (*see* **Note 15)**. Once you have realigned the stack, start data acquisition for a duration appropriate for the speed of action of the drug applied (typically ~20-30 min). After you are done with the drug application, perfuse the slice again with the standard extracellular solution, stop data acquisition, realign your stack if necessary and start the final washout data acquisition session using the same acquisition parameters as before.

11. For imaging of microglial directed motility, in which processes approach an ATP source or site of laser-induced tissue damage, stacks of ~21-31 slices at 2 μm depth intervals are acquired every 30 s. The recommended image acquisition parameters are: 512x512 pixels, 2 frame averages, 1 μsec pixel dwell time (see **Note 13**). Ablation of a small volume of tissue ('laser damage') is performed by illuminating a ~5 μm radius spot with the same 2-photon laser wavelength as is employed for imaging microglial processes but with the laser intensity increased 30-fold and the pixel dwell time increased to 100 μsec. Alternatively, on some systems (such as the Zeiss 710 and 780) it is possible to use a visible confocal laser for photo-ablation. To induce microglial directed motility in response to an ATP (or ADP) source, carefully insert a glass pipette (3-4 MΩ resistance measured in ACSF) filled with 1 mM ATP and a fluorescent dye (eg. Alexa Fluor-488) into the slice (~50-100 μm depth), locate the pipette tip in the centre of the region of interest (use the DIC image to see the pipette) (Fig. 2A) and puff gently to release a small volume of liquid at <10 p.s.i. (*see* **Note 16)**. Immediately start imaging with the recommended image acquisition parameters as above for a total time of ~20-30 min or for as long as it takes for the directed motility to complete (Fig. 2B).

3.3 Offline analysis of microglial dynamics

Microglial motility is a broadly used term, which can refer to movement at the level of individual processes, or describe an entire cell's relocation, including its soma. Two-photon imaging, either *in vivo* or *in situ*, is by now a well-established technique. However, defining the right metrics for quantifying microglial movements is not trivial, and analysing the data often involves tailored, custom-made analysis scripts. Depending on the research focus, various groups have acquired and quantified data in different ways, which can hamper a direct and quantitative comparison between different studies. Broadly, these can be classified into [i] whole-cell metrics such as cell volume or surface area to analyse motility changes at the whole-microglial cell level [5, 11-16], and [ii] subcellular-level metrics mostly describing individual process movements such as the rate at which they extend or retract, the number of branch points, or considering movements or relocations of cell somata [1, 2, 12, 13, 17-22]. To simplify image analysis, most studies analyse data after making a maximum intensity projection from 3D image stacks to obtain a 2D image. This converts volumes into areas, and more accurate results can be achieved by performing a 3D analysis [23, 24]. This however requires fast imaging and considerably more computing power, especially if the analysis is not performed on individual processes but at the whole-cell level.

Here, we describe a step-by-step protocol to analyse microglial surveillance, ramification and directed motility in 2D projected time-lapse movies (x, y, t), which requires the use of Image J (or Fiji) and MATLAB. Programmes are available at https://github.com/AttwellLab/Microglia, but see also [24] for an alternative.

1. Image pre-processing: This step is required for any kind of analysis of the images. Noise is removed by applying filters, and small drift, unavoidable in long time-lapse recordings, is corrected. To do so, it is recommended to open the raw images in ImageJ (or Fiji) and apply filters for background, bleach and drift corrections (*see* **Note 17**). Recommended filters in Fiji are: despeckle, median filter, subtract background, correct 3D drift, bleach correction.
2. Digital cell extraction: For microglial surveillance and ramification analysis it is recommended to perform analysis on individual cells. To do so, open the processed images in ImageJ (or Fiji) and extract cells by manually drawing a region of interest (ROI) around them. The selected area should include the cell body and all the processes of that cell (*see* **Note 13**) over the whole duration of the recording. Erase data around that ROI.
3. Binarization: This step usually requires the experimenter's input to evaluate the threshold for binarisation. It is thus highly recommended to be blinded for that step. For thresholding in Fiji, use *RenyiEntropy* and *dark background*.
4. To quantify surveillance and ramification, the resulting movies are processed using custom-written MATLAB scripts as in [5] (https://github.com/AttwellLab/Microglia). Surveillance, sometimes referred to as *baseline motility*, provides a measure of the tissue volume physically screened or surveyed by microglial processes in a given time. It is defined by examining all dynamic structural changes of a microglia between two consecutive time points, by counting all structures being added e.g. due to process extensions, or removed e.g. due to process retractions. In contrast, ramification captures the cell's morphology at a specific time point and is expressed as the ratio of the cell's surface to its volume (in 3D), or of the cell's perimeter to its area (in 2D after obtaining a maximum-intensity projection).

5. To quantify microglial directed motility, it is necessary to first binarize the field of interest, which should ideally include a couple of microglia (~5). The binarized images can then be processed using custom-written MATLAB scripts as in [5] (https://github.com/AttwellLab/Microglia), inspired by the algorithm described by [25]. Briefly, after the experimenter manually clicks on the target of the chemotactic process (i.e. either the tip of the glass pipette containing ATP or the centre of the area damaged with the laser), the algorithm divides the surrounding area into concentric circles with radii at 2 μm intervals (Fig. 3, step 4b), and then segments these circles into 32 radial sectors, thus creating 32 patches between every two consecutive concentric circles. Then, for each frame, starting from the centre, the algorithm searches in every radial sector for the first patch containing >10 'positive' pixels (microglia). The outputs of the algorithm are, for each frame: (i) the distance to the microglial process front in each sector and (ii) the surface area contained within the converging microglial process front (Fig. 3, step 4b).

4. Notes

1. Both isolectin IB4 and tomato lectin can be used to label microglia in acute brain slices. For *in vivo* labelling of microglia, it is recommended to use tomato lectin conjugated to a fluorophore (e.g. DyLight 594) instead of isolectin, as it has been documented to label fine microglial processes better *in vivo* [6].
2. For local drug applications involving a glass pipette inserted into the tissue, it is advantageous to use HEPES-buffered extracellular solution (E2), since the pH inside the pipette solution cannot be set by bubbling the external solution with 5%CO_2 but requires pre-setting with HEPES.

3. The injury caused by brain excision and slicing will eventually activate microglia, which react by changing their morphology. To avoid excessive microglial activation, it is recommended to use slices no longer than 4h after slicing [5, 17].
4. Decent staining is obtained both at room and physiological (37^{o}C) temperatures, however staining quality decreases with the age of the animals and with the degree of myelination. In general, lectin labelling of microglia works well in slices obtained from young rats or mice < 1 month old.
5. Before adding the slice (using a glass or plastic pipette) to the incubation well for labelling, make sure to not pull over too much slicing or storage solution, e.g. by briefly submerging the slice in extracellular solution of choice (E1 or E2) beforehand.
6. The flow rate should be kept constant throughout an experiment and between different experiments, especially when bath-applying drugs to ensure a comparable drug onset time.
7. The laser should be adjusted to the lowest possible power to achieve a decent signal from the cell processes. In our hands, using a Mai Tai Ti:Sapphire laser, around 1.8% of its maximum power at 800 nm, or 6-8% at 920 nm were sufficient, corresponding to around 5 mW and 12 mW at the preparation, respectively, as measured by an optical power meter. Too high a laser power may lead to microglial process retraction, activation and increases bleaching [5, 11, 21, 26].
8. Live cell imaging of microglia in acute hippocampal slices can be performed in brain slices (rat or mouse) pre-stained with a fluorescent lectin or in mouse slices with microglia genetically labelled with a fluorescent tag (eg., Iba1-eGFP). Pre-incubation (~ 20-30 min) of slices with fluorescent isolectin results in staining of both microglia and blood vessels (Fig. 1A). For better results make use of young (~ P12) rat or (~ P15-P30) mouse slices as the myelination occurring at later developmental stages prevents the proper penetration of the lectin into the deeper layers of the tissue. The fluorescent lectin label both the processes

and cell body of healthy ramified microglia (note that strong cell body fluorescence and/or staining of just few thick microglial processes are signs of microglial activation). In general, the genetically labelled microglia have stronger cell body fluorescence as well as better fluorescent coverage of all the microglial processes and can be used at any developmental stage (Fig. 1A). However, staining intensities may vary strongly depending on the microglial promoter.

9. Use of the harp is important to protect against motion artefacts but be careful not to push or destroy the tissue.
10. Proceed rapidly and avoid the use of epi-fluorescence to protect from unnecessary photo-bleaching and microglial activation.
11. It is important to make sure that the microscope operates in complete darkness. It is also important to be cautious while operating high-powered lasers such as those necessary for these experiments.
12. Aim to use as minimal laser power as possible to avoid microglial damage and activation. When using GFP labelled cells an imaging depth of 100-150 μm below the tissue surface can easily achieved, while lectin labelling is normally limited below 100 μm depth. Avoid acquiring images too close to the slice surface (i.e. ignore the first 50-60 μm) where microglial activation will occur more rapidly.
13. An ideal field of view should include 2-4 bright microglia. The cell body of those cells should be in the centre of the imaged stack (with respect to depth) to ensure that all processes are included in the final image (Fig. 1B, C). In our experience, microglial movement is faithfully captured by acquiring consecutive stacks at least every 60 s, ideally every 30s. It might thus be necessary to adjust imaging parameters to reach this sampling rate, depending on the performance and specifications of the microscope and objectives available to you.

14. It is important to perform regular quality tests of the acquired images throughout the scanning as well as at certain experimental time points (e.g. at the end of each imaging session) by checking the time-lapse videos for the following.

a. Motion artefacts during baseline conditions which might be due to poor harp placement, flow rate disturbances and/or temperature fluctuations.

b. Microglial activation (observed as fast process retraction) can originate from the tissue preparation (slicing, craniotomy) or from photobleaching. To reduce microglial activation, make sure that the composition, osmolarity, pH and oxygenation of the slicing and extracellular solutions are correct, and that the solution is freshly prepared and optimally suited to your experimental conditions. It is further recommended to equilibrate the slices in heated extracellular solution in the perfusion chamber at the microscope stage for at least 5-10 min before imaging. Check that the gassing and temperature remain constant throughout the experiment.

c. To reduce photobleaching reduce the intensity of the laser wherever possible (*see* **Note 6**).

15. It is important to correct any drifts of the focused area throughout the imaging experiments in order to facilitate the offline image processing and yield of usable cells. To realign the area of interest, navigate back to the first image stack taken and adjust appropriately (*see* methods step 8) by focusing on the central slice (e.g. slice 11 out of total 21 slices-stack). Use specific structures of the initial stack (e.g. parts of a microglial cell body, blood vessels etc.) as alignment markers and compare their topology with the image to be adjusted. If necessary, change the x, y, z positions until the two images are almost identical.

16. The insertion of the pipette into the slice requires precision and slow motion to minimize tissue damage in the field of interest. To achieve that, it is recommended to carefully insert the pipette in the "approach mode" (where the movement is along the axis of the electrode, if available with your pipette micromanipulator) into the region of interest at an angle of

~ 25-30 degrees. Move the pipette slowly (< 5 μm/s) and apply some positive pressure to avoid clogging of the pipette tip.

17. It is recommended to design an automated image processing routine (e.g. macros in ImageJ) that can be used to preprocess all of the images in a standardised manner.

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Figure Legends

Figure 1. Imaging of fluorescent microglia in acute brain slices.

(A) Maximum intensity projections (MIP) of two-photon image stacks (27 slices, 2 µm step size) showing (left) microglia labelled with isolectin B_4 conjugated to Alexa 594 in an acute brain slice of a P12 rat and (right) genetically labelled microglia in an acute brain slice of a P30 Iba1-EGFP mouse. Note that isolectin B_4 stains both microglia and blood vessels (asterisks). (B) Representative region of interest in the central slice of the image stack shown as a MIP in in the right panel in (A). Arrows indicate microglial cell bodies. (C) Representative slices of a substack of the region of interest in (B) (dotted box) show that the entire microglial cell including all processes is properly incorporated in the z-stack. Note that processes can extend up to 30 µm away from the centrally located cell body.

Figure 2. Imaging of ATP-induced directed motility of microglia in acute brain slices.

(A) Representative DIC and two-photon images of acute cortical brain slices of a 2 month old wild-type mouse: (left) DIC image showing the tip of the pipette inserted at the center of the region of interest; (middle) Two-photon image illustrating the ATP-filled pipette containing 10 µM Alexa Fluor 488; (right) Two-photon image showing isolectin B4 (conjugated to Alexa 594) labelled microglial cells and blood vessels in red. (B) Selected images at different time points (t = 0, 15 and 20 mins) showing the convergence of microglial processes towards the tip of the pipette containing 1mM ATP (green).

Figure 3. Workflow for data acquisition and analysis.

Schematic illustration of the workflow for data acquisition and analysis. 4D stacks (x-y-z-t) are first acquired using a two-photon microscope following the experimental design of choice (either baseline surveillance or targeted motility, see 3.2.9-3.2.11). The stacks are then

preprocessed in ImageJ (or equivalent software) to yield binarised 2D time-lapse movies (x-y-t) that can be easily analysed and quantified. MATLAB scripts are then applied to those to quantify either [a] baseline surveillance or [b] targeted motility to a chemoattractant. Note that, in the latter case, we adapted the method by [25]. Quantification displayed here is the average distance of the microglial process front to the target (left; µm) and the surface of the red polygon delimited by the microglial process front (right; μm^2). In both cases, the time displayed is measured in min.

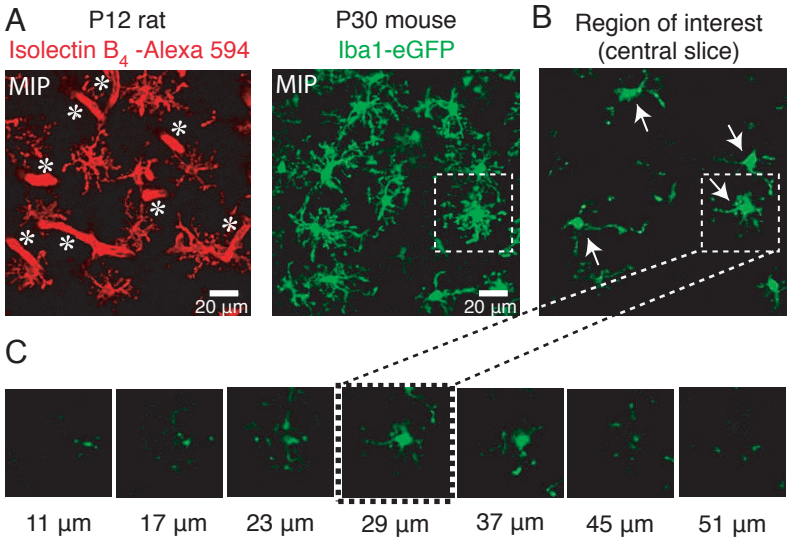

A
P12 rat
Isolectin B4 -Alexa 594
MIP
20 µm
P30 mouse
Iba1-eGFP
MIP
20 µm
B
Region of interest
(central slice)
C
11 µm
17 µm
23 µm
29 µm
37 µm
45 µm
51 µm

Figure 1

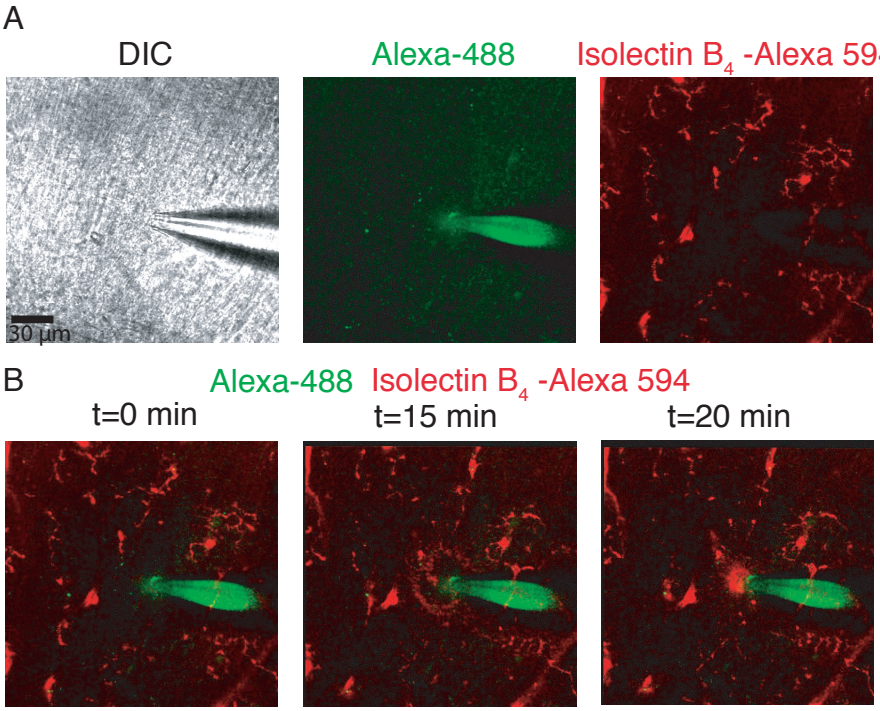
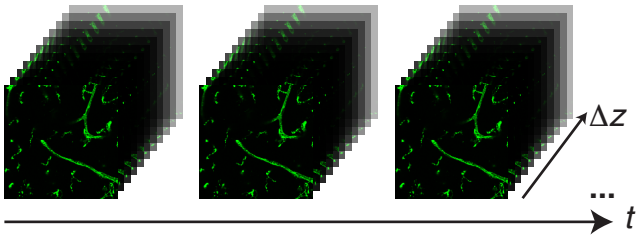

A
DIC
Alexa-488
Isolectin B4 -Alexa 594
30 µm
B
Alexa-488 Isolectin B4 -Alexa 594
t=0 min
t=15 min
t=20 min

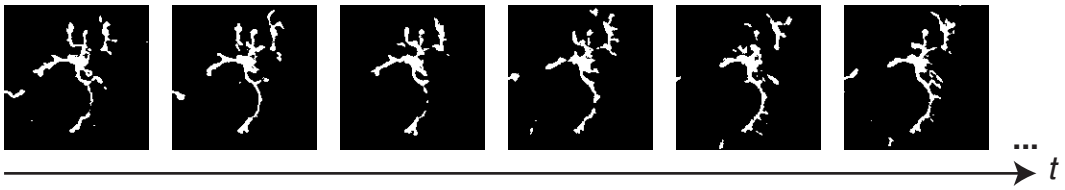
Figure 2

1. Data acquistion
(4D stacks x-y-z-t)

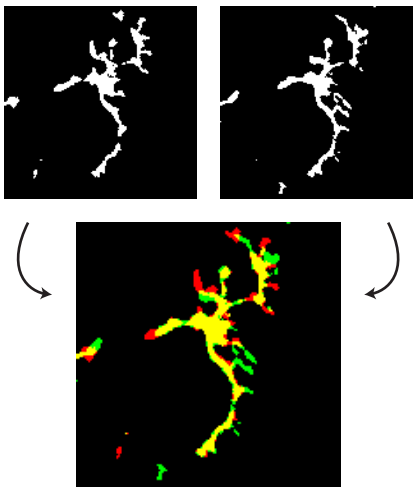


2. Preprocessing
using ImageJ

3. Binarised 2D time-lapse movies (x-y-t)

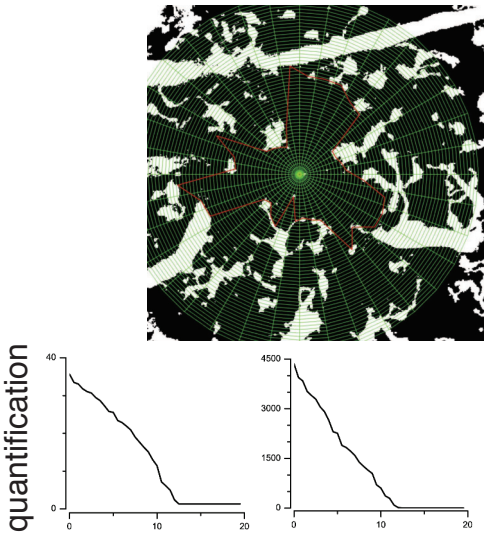


4a. MATLAB quantification
of ramification and motility



retractions vs extensions

4b. MATLAB quantification
of chemotaxis



time

Figure 3