







# MotilA – A Python pipeline for the analysis of microglial fine process motility in 3D time-lapse multiphoton microscopy data

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## Summary

*MotilA* is an open-source Python pipeline for quantifying microglial fine-process motility in 3D time-lapse two-channel fluorescence microscopy. It was developed for high-resolution *in vivo* multiphoton imaging and supports single-stack and batch analyses. The workflow performs sub-volume extraction, optional registration/unmixing, z-projection, segmentation, and pixel-wise change detection to compute the turnover rate (TOR). The code is platform independent, documented with tutorials and example datasets, and released under GPL-3.0.

## Statement of need

Microglia are immune cells of the central nervous system and continuously remodel processes to survey brain tissue and respond to pathology (M. Fuhrmann et al., 2010; Nimmerjahn et al., 2005; Prinz et al., 2019; Tremblay et al., 2010). Quantifying this subcellular motility is important for studies of neuroinflammation, neurodegeneration, and synaptic plasticity. Current practice in many labs relies on manual or semi-manual measurements in general-purpose tools such as Fiji/ImageJ or proprietary software (Carl Zeiss Microscopy GmbH, Accessed 2025; Schindelin et al., 2012). These procedures are time consuming, hard to reproduce, focus on single cells, and are sensitive to user bias (Brown, 2017; Wall et al., 2018). There is no dedicated, open, and batch-capable solution tailored to this task.

*MotilA* fills this gap with an end-to-end, reproducible pipeline for 3D time-lapse two-channel imaging. It standardizes preprocessing, segmentation, and motility quantification and scales from individual stacks to large experimental cohorts. Unlike Fiji/ImageJ macros or proprietary packages, *MotilA* provides a fully automated non-interactive workflow in Python that applies identical parameters across datasets, logs all intermediate steps, and avoids user-dependent adjustments. This ensures reproducible, bias-minimized, and scalable processing of large 3D time-lapse datasets, including optional motion correction and spectral unmixing. Although optimized for microglia, the approach generalizes to other motile structures that can be reliably segmented across time.

To clarify *MotilA*'s novelty relative to existing analysis approaches, the following table summarizes key differences between *MotilA*, Fiji/ImageJ, and ZEISS ZEN:

**Table 1.** Comparison of *MotilA* with commonly used alternatives for microglial motility analysis.

Feature	Fiji/ImageJ	ZEISS ZEN	MotilA
<b>Automation</b>	Limited. User-recorded macros; complex workflows often require manual steps and must be split across several macros.	None. Full user interaction required.	Full. End-to-end non-interactive workflow.
<b>Batch processing</b>	Limited. Macros can process several files in one folder, but they cannot navigate nested directory structures or manage multi-step 3D multi-channel time-series pipelines.	None. Each dataset processed manually.	Full. Metadata-driven cohort processing.
<b>Reproducibility</b>	Moderate. Requires complete manual logging; interactive tuning reduces reproducibility.	Low. Manual adjustments introduce strong user bias.	High. Full parameter logging and deterministic runs.
<b>Scalability</b>	Low. Full-stack RAM loading; no chunked I/O for large 3D data.	Low-medium. Efficient viewing but no automated processing for large time-lapse datasets.	High. Chunked I/O for multi-gigabyte 3D two-channel stacks.
<b>Open-source</b>	Yes (GPL-3.0).	No (proprietary).	Yes (GPL-3.0).

## Implementation and core method

Input is a 5D stack in TZCYX or TZYX order, where T is time, Z is depth, C is channel, and YX are spatial dimensions. For each time point, *MotilA* extracts a user-defined z-sub-volume, optionally performs 3D motion correction and spectral unmixing, and computes a 2D maximum-intensity projection to enable interpretable segmentation. After thresholding, the binarized projection  $B(t_i)$  is compared with  $B(t_{i+1})$  to derive a change map

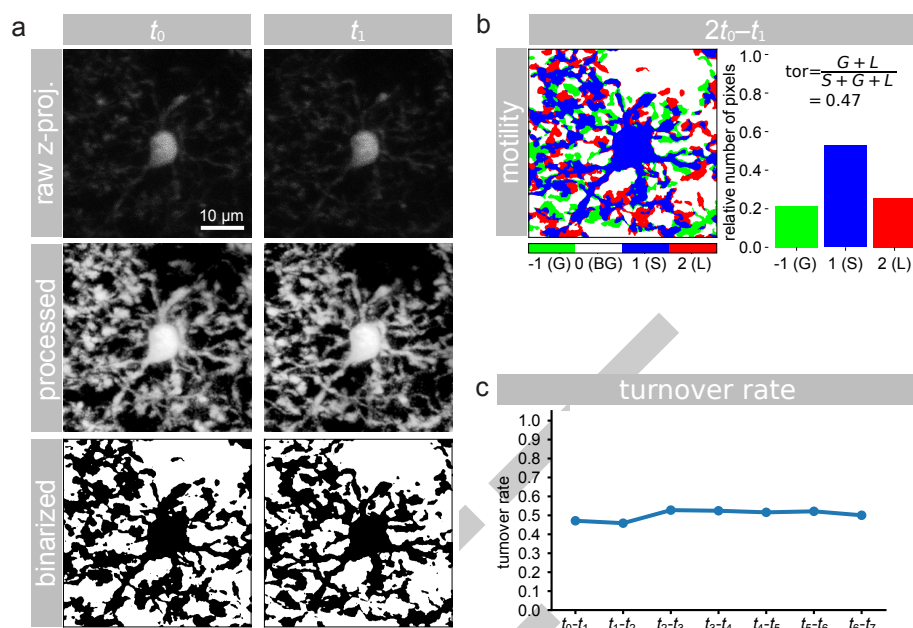
$$\Delta B(t_i) = 2B(t_i) - B(t_{i+1}).$$

Pixels are classified as stable “S” ( $\Delta B = 1$ ), gained “G” ( $\Delta B = -1$ ), or lost “L” ( $\Delta B = 2$ ). From these counts, the turnover rate is defined as

$$TOR = \frac{G + L}{S + G + L},$$

representing the fraction of pixels that changed between consecutive frames. This pixel-based strategy follows earlier microglial motility work (M. Fuhrmann et al., 2010; Nebeling et al., 2023) while providing a fully automated and batchable implementation with parameter logging and diagnostics.

The pipeline exposes options for 3D or 2D registration, contrast-limited adaptive histogram equalization, histogram matching across time to mitigate bleaching, and median or Gaussian filtering (Pizer et al., 1987; Virtanen et al., 2020; Walt et al., 2014). Results include segmented images, G/L/S/TOR values, brightness and area traces, and spreadsheets for downstream statistics. Memory-efficient reading and chunked processing of large TIFFs are supported via Zarr (Miles et al., 2025).



**Figure 1:** Example analysis with MotiLA. **a)** z-projected microglial images at two consecutive time points ( $t_0$ ,  $t_1$ ), shown as raw, processed, and binarized data. **b)** pixel-wise classification of gained (G), stable (S), and lost (L) pixels used to compute the turnover rate (TOR). **c)** TOR values across time points from the same dataset, illustrating dynamic remodeling of microglial fine processes.

## Usage

*MotiLA* can be called from Python scripts or Jupyter notebooks. Three entry points cover common scenarios: `process_stack` for a single stack, `batch_process_stacks` for a project folder organized by dataset identifiers with a shared metadata sheet, and `batch_collect` to aggregate metrics across datasets. All steps write intermediate outputs and logs to facilitate validation and reproducibility. *MotiLA*'s GitHub repository provides tutorials and an example dataset to shorten onboarding.

## Applications and scope

*MotiLA* has been applied to quantify microglial process dynamics in several *in vivo* imaging studies and preprints (Crux et al., 2024; F. Fuhrmann et al., 2024; Gockel et al., 2025). Typical use cases include baseline surveillance behavior, responses to neuroinflammation or genetic perturbations, and deep three-photon imaging where manual analysis is impractical. The binarize-and-compare principle can in principle be adapted to other structures such as dendrites or axons when segmentation across time is robust.

## Limitations

Using 2D projections simplifies processing but sacrifices axial specificity and can merge overlapping structures. Segmentation quality determines accuracy and can be affected by vessels, low signal-to-noise ratios, or strong intensity drift. The current spectral unmixing is a simple subtraction; advanced approaches may be needed for some fluorophores. *MotiLA* targets pixel-level process motility rather than object-level tracking or full morphometry.

## Example dataset

The repository includes two *in vivo* two-photon stacks from mouse frontal cortex formatted for use with *MotilA* (Gockel et al., 2025). Each stack contains eight time points at five-minute intervals, two channels for microglia and neurons, and approximately sixty z-planes at one micrometer steps in a field of view of about 125 by 125 micrometers. The example reproduces the full analysis, including projections, segmentation, change maps, brightness traces, and TOR over time, and serves as a template for cohort-level workflows.

## Availability

Source code, documentation, tutorials, and issue tracking are hosted at: <https://github.com/FabrizioMusacchio/motila>. The software runs on Windows, macOS, and Linux with Python 3.9 or newer and standard scientific Python stacks. It is released under GPL-3.0, and contributions via pull requests or issues are welcome.

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