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

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Software

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Summary

MotilA is an open-source Python pipeline for quantifying microglial fine-process motility in 4D (TZYX) or 5D (TZCYX) time-lapse fluorescence microscopy data, supporting both single-channel and two-channel acquisition. It was developed for high-resolution *in vivo* multiphoton imaging and supports both single-stack and cohort-scale batch analyses. The workflow performs sub-volume extraction, optional registration and spectral unmixing, a maximum-intensity projection along the Z-axis, segmentation, and pixel-wise change detection to compute the turnover rate (TOR). *MotilA* specifically targets pixel-level process motility rather than object tracking or full morphometry. 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 their 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
Automation	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.
Batch processing	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.
Reproducibility	Moderate. Requires complete manual logging; interactive tuning reduces reproducibility.	Low. Manual adjustments introduce strong user bias.	High. Full parameter logging and deterministic runs.
Scalability	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.
Open-source	Yes (GPL-3.0).	No (proprietary).	Yes (GPL-3.0).

Implementation and core method

Input is a 5D stack in TZCYX or a 4D stack in TZYX order, where T is time, Z is depth, C is channel, and YX are spatial dimensions. *MotilA* does not assume a fixed channel order. Users specify which channel contains microglia and which, if present, provides a structural reference signal, such as a neuronal label. Although the reference channel does not enter the motility computation, it is commonly acquired in microglial imaging because it offers stable features that support robust pre-processing registration of the 3D stack before it is passed to *MotilA*. The additional channel may also be used for optional spectral unmixing in the presence of bleed-through.

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 along the Z-axis 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; van der Walt et al., 2014; Virtanen et al., 2020). 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).

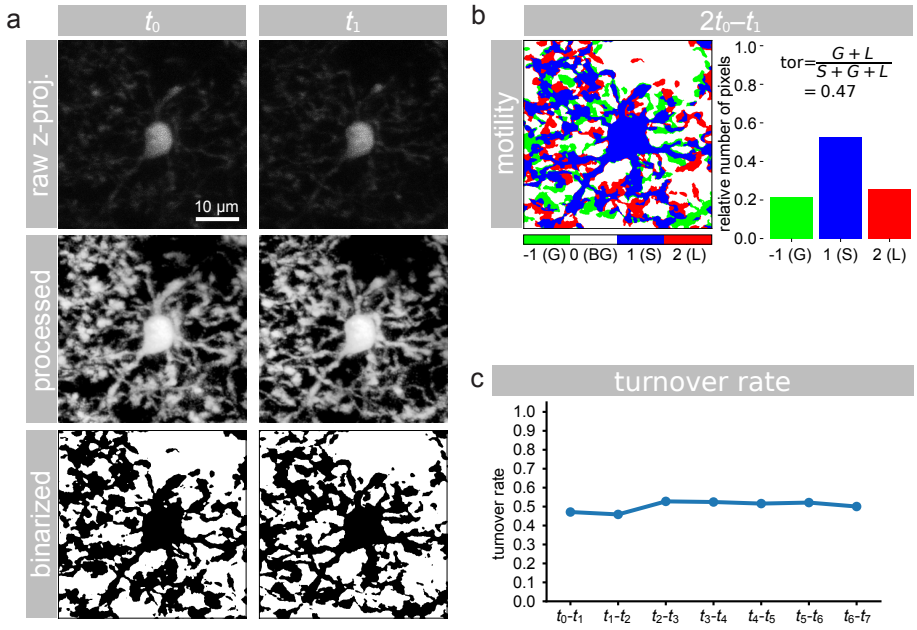

Figure 1: Example analysis with MotilA. **a)** z-projected microglial images at two consecutive time points (t_0, t_1), shown as raw, processed, and binarized data. **b)** pixel-wise classification of gained (G), stable (S), and lost (L) pixels used to compute the turnover rate (TOR). **c)** TOR values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

MotilA quantifies microglial process motility using 2D maximum-intensity projections rather than fully volumetric analysis. This choice reflects practical constraints of two-photon microglial

imaging: axial resolution degrades with depth, producing elongated point-spread functions and reduced contrast along Z, which makes reliable voxel-wise 3D segmentation of thin processes difficult. Z-projection increases effective signal per pixel and follows established practice in earlier microglial motility studies (see, e.g., M. Fuhrmann et al. (2010); Nebeling et al. (2023)), but necessarily sacrifices axial specificity and may merge structures that overlap along Z. Users are therefore advised to select sub-volumes with minimal axial overlap.

Segmentation quality critically determines the accuracy of motility estimates and can be affected by blood vessels, low signal-to-noise ratios, and intensity drift over time. The current spectral unmixing is implemented as a simple subtraction and may be insufficient for fluorophores with complex spectral overlap. Finally, *MotilA* focuses on pixel-level process motility rather than object-level tracking or full morphological reconstruction. Fully three-dimensional motility analysis would require volumetric segmentation and substantially higher computational resources and is beyond the scope of the current version.

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. An example dataset used for demonstration and testing purposes is available via Zenodo (Gockel et al., 2025) and described in the documentation.

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