

¹ MotilA – A Python pipeline for the analysis of
² microglial fine process motility in 3D time-lapse
³ multiphoton microscopy data

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⁷ Summary

⁸ *MotilA* is an open-source Python pipeline for quantifying microglial fine-process motility in
⁹ 4D (TZYX) or 5D (TZCYX) time-lapse fluorescence microscopy data, supporting both single-
¹⁰ channel and two-channel acquisition. It was developed for high-resolution *in vivo* multiphoton
¹¹ imaging and supports both single-stack and cohort-scale batch analyses. The workflow
¹² performs sub-volume extraction, optional registration and spectral unmixing, a maximum-
¹³ intensity projection along the Z-axis, segmentation, and pixel-wise change detection to compute
¹⁴ the turnover rate (TOR). *MotilA* specifically targets pixel-level process motility rather than
¹⁵ object tracking or full morphometry. The code is platform independent, documented with
¹⁶ tutorials and example datasets, and released under GPL-3.0.

¹⁷ Statement of need

¹⁸ 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](#); [Nimmer-
jahn 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. Cur-
rent 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.

²² *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.

³⁶ To clarify *MotilA*'s novelty relative to existing analysis approaches, the following table summa-
³⁷ rizes key differences between *MotilA*, Fiji/ImageJ, and ZEISS ZEN:

³⁸ **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).

³⁹ Implementation and core method

⁴⁰ 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 ⁴¹ specify which channel contains microglia and which, if present, provides a structural reference ⁴² signal, such as a neuronal label. Although the reference channel does not enter the motility ⁴³ computation, it is commonly acquired in microglial imaging because it offers stable features ⁴⁴ that support robust pre-processing registration of the 3D stack before it is passed to *MotilA*. ⁴⁵ The additional channel may also be used for optional spectral unmixing in the presence of ⁴⁶ bleed-through.

⁴⁷ For each time point, *MotilA* extracts a user-defined z-sub-volume, optionally performs 3D ⁴⁸ motion correction and spectral unmixing, and computes a 2D maximum-intensity projection ⁴⁹ along the Z-axis to enable interpretable segmentation. After thresholding, the binarized ⁵⁰ projection $B(t_i)$ is compared with $B(t_{i+1})$ to derive a change map

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

⁵² Pixels are classified as stable “S” ($\Delta B = 1$), gained “G” ($\Delta B = -1$), or lost “L” ($\Delta B = 2$). ⁵³ From these counts, the turnover rate is defined as

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

⁵⁴ representing the fraction of pixels that changed between consecutive frames. This pixel-based ⁵⁵ strategy follows earlier microglial motility work (M. Fuhrmann et al., 2010; Nebeling et al., ⁵⁶ 2023) while providing a fully automated and batchable implementation with parameter logging ⁵⁷ and diagnostics.

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

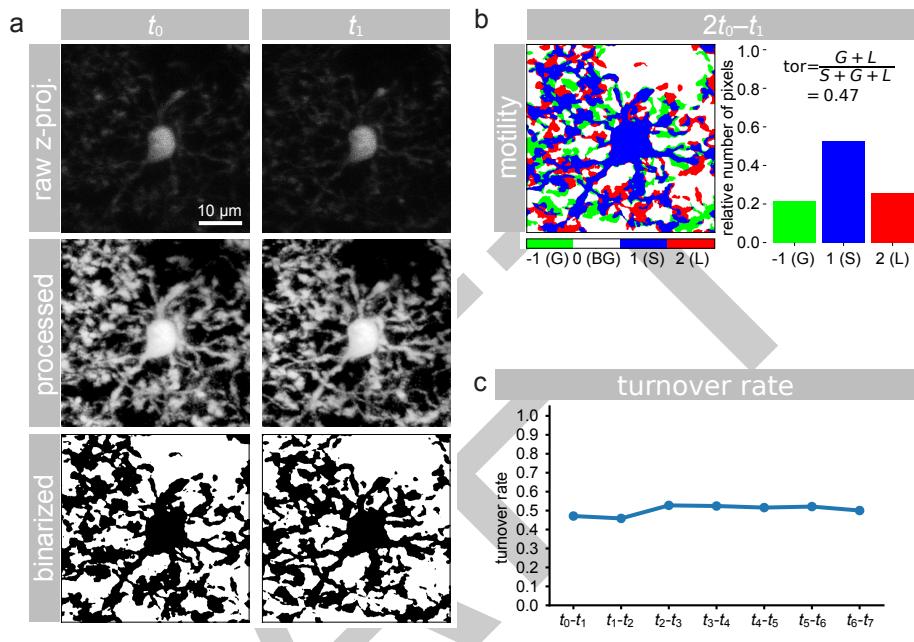


Figure 1: Example analysis with *MotilA*. **a)** z-projected microglial images at two consecutive time points (t_0, t_1), shown as raw, processed, and binarized data. **b)** pixel-wise classification of gained (G), stable (S), and lost (L) pixels used to compute the turnover rate (TOR). **c)** TOR values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

Usage

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

Applications and scope

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

Limitations

78 Using 2D projections simplifies processing but sacrifices axial specificity and can merge
 79 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.

Using 2D Z-projections confines motility quantification to the XY plane, but this reflects practical constraints of two-photon microglial imaging. Axial resolution degrades with imaging depth, yielding elongated point-spread functions and reduced contrast along Z, which makes voxel-wise 3D segmentation of thin microglial processes unreliable. Maximum-intensity projection increases effective signal per pixel and follows established practice in earlier microglial motility work (see, e.g., M. Fuhrmann et al. (2010); Nebeling et al. (2023)). This approach necessarily sacrifices axial specificity and can merge structures that overlap in Z, particularly in densely populated regions, which are best avoided by selecting a sub-volume with minimal axial overlap. Fully 3D motility analysis would require volumetric segmentation, morphological reconstruction, and substantially higher memory and computational resources and is therefore out of scope for the current version of *MotilA*.

Beyond the inherent limitations of Z-projection, segmentation quality critically affects accuracy and can be influenced by blood vessels, low signal-to-noise ratios, and strong intensity drift across time. The current spectral unmixing is implemented as a simple subtraction and may be insufficient for fluorophores with complex spectral overlap. *MotilA* targets pixel-level process motility rather than object-level tracking or full morphometry, and its interpretability depends on reliable and consistent segmentation across frames.

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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