

# <sup>1</sup> 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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## <sup>7</sup> Summary

<sup>8</sup> *MotilA* is an open-source Python pipeline for quantifying microglial fine-process motility in <sup>9</sup> 3D time-lapse two-channel fluorescence microscopy. It was developed for high-resolution <sup>10</sup> *in vivo* multiphoton imaging and supports single-stack and batch analyses. The workflow <sup>11</sup> performs sub-volume extraction, optional registration/unmixing, z-projection, segmentation, <sup>12</sup> and pixel-wise change detection to compute the turnover rate (TOR). The code is platform <sup>13</sup> independent, documented with tutorials and example datasets, and released under GPL-3.0.

## <sup>14</sup> Statement of need

<sup>15</sup> Microglia are immune cells of the central nervous system and continuously remodel processes <sup>16</sup> 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 <sup>17</sup> important for studies of neuroinflammation, neurodegeneration, and synaptic plasticity. Current <sup>18</sup> practice in many labs relies on manual or semi-manual measurements in general-purpose tools <sup>19</sup> such as Fiji/ImageJ or proprietary software ([Carl Zeiss Microscopy GmbH, Accessed 2025](#); <sup>20</sup> [Schindelin et al., 2012](#)). These procedures are time consuming, hard to reproduce, focus on <sup>21</sup> single cells, and are sensitive to user bias. ([Brown, 2017](#); [Wall et al., 2018](#)). There is no <sup>22</sup> dedicated, open, and batch-capable solution tailored to this task. <sup>23</sup>

<sup>24</sup> *MotilA* fills this gap with an end-to-end, reproducible pipeline for 3D time-lapse two-channel <sup>25</sup> imaging. It standardizes preprocessing, segmentation, and motility quantification and scales <sup>26</sup> from individual stacks to large experimental cohorts. Although optimized for microglia, the <sup>27</sup> approach generalizes to other motile structures that can be reliably segmented over time. <sup>28</sup>

## <sup>28</sup> Implementation and core method

<sup>29</sup> Input is a 5D stack in TZCYX or TZYX order, where T is time, Z is depth, C is channel, <sup>30</sup> and YX are spatial dimensions. For each time point, *MotilA* extracts a user-defined z-sub- <sup>31</sup> volume, optionally performs 3D motion correction and spectral unmixing, and computes a 2D <sup>32</sup> maximum-intensity projection to enable interpretable segmentation. After thresholding, the <sup>33</sup> binarized projection  $B(t_i)$  is compared with  $B(t_{i+1})$  to derive a change map

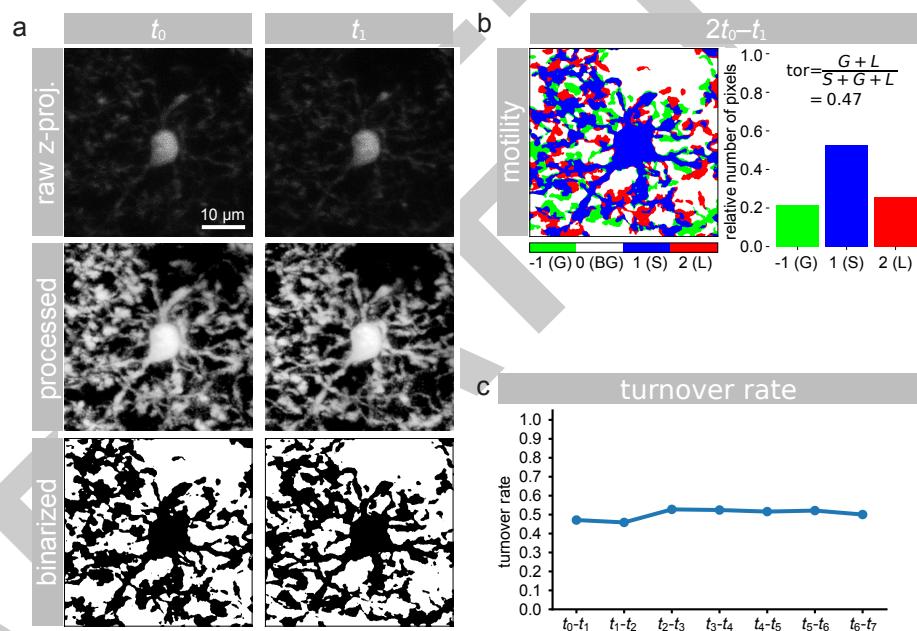
$$\Delta B(t_i) = 2B(t_i) - B(t_{i+1}).$$

<sup>34</sup> Pixels are classified as stable “S” ( $\Delta B = 1$ ), gained “G” ( $\Delta B = -1$ ), or lost “L” ( $\Delta B = 2$ ). <sup>35</sup> 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.

## 46 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.

## 53 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

57 perturbations, and deep three-photon imaging where manual analysis is impractical. The  
58 binarize-and-compare principle can in principle be adapted to other structures such as dendrites  
59 or axons when segmentation across time is robust.

## 60 Limitations

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

## 66 Example dataset

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

## 73 Availability

74 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  
75 or newer and standard scientific Python stacks. It is released under GPL-3.0, and contributions  
76 via pull requests or issues are welcome.  
77

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