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Spatial mapping reveals multi-step pattern of wound healing in *Physarum polycephalum*

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Abstract

Wounding is a severe impairment of function, especially for an exposed organism like the network-forming true slime mould *Physarum polycephalum*. The tubular network making up the organism's body plan is entirely interconnected and shares a common cytoplasm. Oscillatory contractions of the enclosing tube walls drive the shuttle streaming of the cytoplasm. Cytoplasmic flows underlie the reorganization of the network for example by movement toward attractive stimuli or away from repellants. Here, we follow the reorganization of *P. polycephalum* networks after severe wounding. Spatial mapping of the contraction changes in response to wounding reveal a multi-step pattern. Phases of increased activity alternate with cessation of contractions and stalling of flows, giving rise to coordinated transport and growth at the severing site. Overall, severing surprisingly acts like an attractive stimulus enabling healing of severed tubes. The reproducible cessation of contractions arising during this wound-healing response may open up new venues to investigate the biochemical wiring underlying *P. polycephalum*'s complex behaviours.

Keywords: slime mould, behaviour, transport network, image analysis

S Supplementary material for this article is available online

(Some figures may appear in colour only in the online journal)

1. Introduction

Simple organisms like fungi and slime moulds are able to display complex behaviours. This is surprising given that their network-like body plan lacks any central organizing centre. The slime mould *Physarum polycephalum* has emerged as a model system to study the complex dynamics these organisms use to adapt to their environment. The organism has been shown to find the shortest path through a maze [1] and connect food sources in an efficient and at the same time robust network comparable to man-made transport networks [2]. Furthermore, the slime mould distributes its body mass among several resources to obtain an optimal diet [3] and is able to anticipate recurring stimuli [4].

P. polycephalum is a true slime mould that forms a plasmodial network. Nuclei keep on dividing without forming cell walls, which results in a syncytial web-like network. The cytoplasm within this tubular network flows back and forth in a shuttle flow [5]. These cytoplasmic flows are driven by cross-sectional contractions of the actin-myosin meshwork lining the gel-like tube walls [6]. Flows are organized across the entire network in a peristaltic wave of contractions that matches organism size [7]. Flows generated in the organism are optimized for transport as contractions increase the effective dispersion of particles way beyond molecular diffusivity by a mechanism called Taylor dispersion [8].

P. polycephalum adapts its network-like morphology to its environment by chemotaxis [9–11]. Here, stimulants are classified by being an attractant or a repellant depending on the organism's response to migrate toward or away from the stimulant. Stimulants have also been shown to affect

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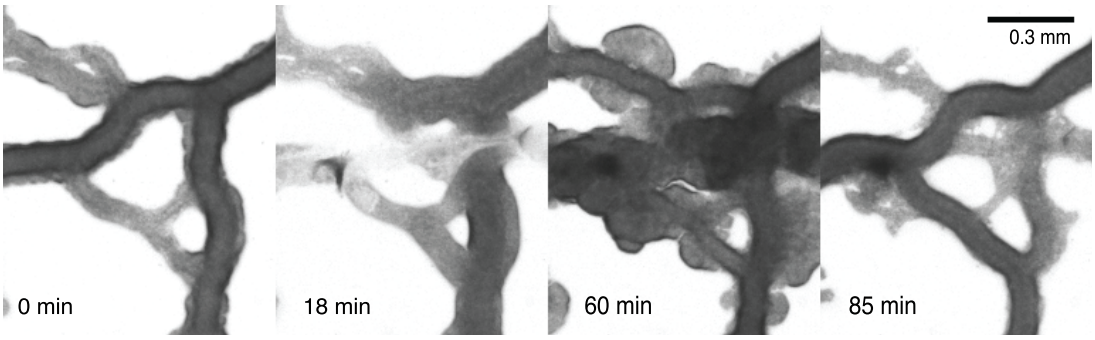

Figure 1. Wound healing process in *P. polycephalum* illustrated at four time points using bright field images. The cut occurred at 18 min and the fan grown at cut site reached its maximal size at 60 min. The network morphology was restored after 85 min.

cross-sectional contractions organism-wide by an increase in their frequency and amplitude for an attractant or a decrease for a repellant [12, 13]. A variety of chemical stimuli have been discussed for *P. polycephalum*, with glucose being a prominent attractant and salts like NaCl being effective repellants [14–16]. Temperature [17, 18] and light [19, 20] have also been found to act as stimulants that trigger organism-wide restructuring of the transport networks' morphology. In fact, the cytoplasmic flows themselves serve as the medium by which stimuli pervade the organism [21].

A lot less is known about the impact of mechanical perturbations on the organism. In its natural habitat the slime mould suffers predation from grazing invertebrates causing severing that disrupts the transport network and its cytoplasmic flows. In experiments it has been found that quickly stretching a strand to 10–20% of its length while keeping it intact increases the amplitude of oscillations [22]. Excising a single strand from a plasmodial network has been observed to lead to a roughly 20 minute cessation of contractions in the strand until recovery [23]. This phenomenon was not observed for strands excised from the growing fan region of the slime mould resulting in speculations about the motive force being limited to the fan only. Yet, the cessation of contractions turned out to be hard to reproduce, see [24] and references therein. Among these discordant observations what remains established is local gelation of cytoplasmic flows upon touch without severing the organism [25]. Despite the limited knowledge, wounding the organism by severing the network is part of daily laboratory routines and an eminent perturbation in natural habitat.

Here, we investigate *P. polycephalum*'s dynamics during wound healing following the quick and complete severing of a tube within the organism's network. We follow the process of wound healing across the individual's entire body, over the course of one hour after severing. The exemplary quantitative analysis of organism-wide contractions reveals a stepwise response spanning four different states. Briefly after severing, the contractions are often marked by an increase in amplitude and frequency, followed by a several minutes long cessation of contractions and stalling of cytoplasmic flows. This resting state is terminated by a sudden restart of vigorous contractions as the severed tube re-fuses. The vigorous state then transitions into a state of network-spanning contractions and continuous fan growth at the wounding site until the organism reverts back to pre-stimulus dynamics. Timing and significance of individual steps varies with the severity of cutting and cutting site location within the network. For example, stalling is found to be less pronounced when the network is cut in fan-like region. Overall, quick and complete severing triggers a response pattern with characteristics of the response to an attractive stimulus, including an increase in amplitude and frequency and net movement to stimulus site, see figure 1. The reproducibility of stalling clarifies earlier contradictions and at the same time opens new avenues to investigate the biochemical dynamics behind the highly coordinated actomyosin contractions underlying *P. polycephalum*'s arguably fascinating dynamics.

2. Methods

2.1. Culturing and data acquisition

The plasmodium is prepared from microplasmodia grown in liquid medium. The recipe for the medium is inspired by [26], see section S1. The advantage of this method over growing the plasmodium on oat flakes or bacteria is the ability to precisely control the nutritional state and amount of the organism. Also, plasmodia grown this way are free from oat flake residues or vacuoles containing food, which provides a cleaner sample for imaging. To prepare the plate for imaging, 0.2–0.5 ml of the microplasmodia grown in a shaking culture at 30 °C are transferred to an 1.5% agar plate and stored in a closed, but not sealed, dish in the dark. After 12–24 h, the microplasmodia fuse into a single plasmodium. The plasmodium is ready for imaging when there are no visible traces of liquid medium and the organism assumed its characteristic network shape, which usually occurs up to 36 h after plating.

The imaging is performed with a Zeiss Axio Zoom V.16 microscope, equipped with a Zeiss PlanNeoFluar $1x/0.25$ objective and a Hamamatsu ORCA-Flash 4.0 digital camera. A green filter (550/50 nm) is placed over the transmission light source of the microscope to diminish *P. polycephalum*'s response to the light, and a humidity chamber prevents the sample from drying out. The acquisition of the images is done in Zeiss ZEN 2 (Blue Edition) software with bright-field setting. During the acquisition, the illumination of the sample is kept constant, and an image is taken every 3 s. The plasmodium is imaged for $\sim$1 h before the application of the mechanical stimulus to allow for the accommodation to the light [10]. The stimulus is applied manually, using a microinjection needle with a blunt tip. The needle tip is held above the

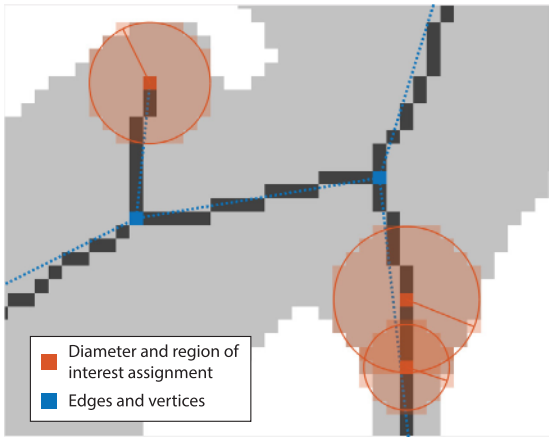

Figure 2. Scheme of intensity and diameter data extraction based on *P. polycephalum* bright field images. The light grey area depicts the network mask based on the bright field images. Dark grey lines represent the network skeleton and the corresponding topology is shown in blue. Each pixel of the skeleton acts as a reference point for data derived during the analysis. The diameter is set as the distance from the reference point to the next non-mask pixel. The intensity is calculated by averaging individual pixel intensities over a corresponding disk (red).

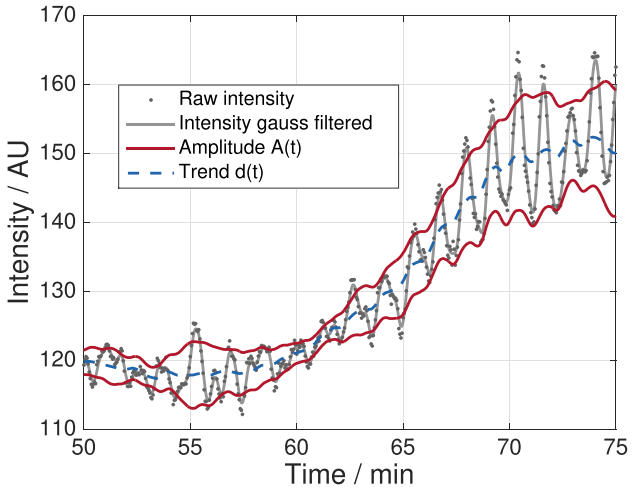

Figure 3. Derivation of oscillation specific parameters, i.e. amplitude $A(t)$, frequency $f(t)$ and trend $d(t)$, from single pixel time series. The trend is calculated using a moving average with a kernel width of 200 s. Intensity is filtered with a Gaussian of width 39 s. Amplitude and frequency are calculated from the absolute value and angle of the complex-valued analytic signal, respectively.

surface of the agar at a small angle and quickly dragged across the chosen plasmodial tube. The cut is severe and complete if the two parts of the tube separate completely. The plasmodium is then further imaged for more than 1 h.

Using microplasmodia is so far the optimal way of obtaining non-severed networks, where the size and nutritional state are reproducible. However, there are challenges during the imaging that decrease the reproducibility of the experiment. In particular, plasmodia are highly motile and change their morphology accordingly. Furthermore, the organism tends to develop very large foraging fronts, which are not a suitable input for the presented comprehensive data analysis as they lack network characteristics. Lastly, the microscope light can act as stimulus [2, 19, 20], and even the green-filtered low-intensity illumination may cause the network to respond and change its behaviour to escape the imaging region. These challenges combined make the reproducibility and required stability of the network morphology over time challenging.

2.2. Comprehensive network-based contraction analysis

To quantify contraction dynamics we analyse bright field recordings in two different ways: for two morphologically static networks (see E2 and E3 in the experiment list) we perform an exhaustive network-based analysis as outlined in the following (see figures 5 and S4, available online at stacks.iop.org/JPhysD/50/434005/mmedia). For the additional 19 specimen, which alter their network morphology dramatically over the course of the experiment, we analyse kymographs along static parts of the network as described in details in section S3 (see exemplary E1 and mov. S5).

Images recorded as a time series are processed as 8-bit uncompressed TIFs. At first every image is processed separately, then the results are stitched together, largely following [7], and lastly the collective is analysed. On every single image, background is removed with the rolling-ball method. Then the image is used to create a mask, a binary image, with an intensity threshold that separates the network from the background. The mask is enhanced further, i.e. only the biggest structure is considered, small holes are filled and single-pixel edges are smoothed. Subsequently, the resulting mask is used as a template for extracting the network's skeleton with a thinning method. In the skeletonized mask each pixel can be understood as a data point representing local intensity and diameter (see figure 2). Local diameter is calculated as the largest fitting disk radius around the point within the mask. Within this disk the average intensity is computed and saved as intensity at the considered data point. Intensity and diameter anti-correlate due to the optical density of the slime mould and can therefore be used interchangeably considering Beer-Lambert law. Individual data points are attributed to a specific network branch of the network skeleton. To represent network topology, the network is broken down into vertices and edges where vertices describe pixel positions of branching points and edges represent two connected vertices. Each edge then acts as a parent for one specific branch. In this sense edges are abstracted simple connections and branches represent pixel-based resolution of a tube.

After the network is extracted in space, the edges, vertices, diameters, and intensities are concatenated in time. To map intensity and diameter over time, a reference image is used, usually from an early time point. For every data point the shortest distance to any pixel in the reference image is calculated. This gives a quasi-static $(x, y, t) \rightarrow$ (intensity, diameter) dataset, i.e. the topology and vertex positions stay the same, but intensity and diameter can vary. This is justified as long as growth of the organism and vertex movement is minimal. The oscillatory behaviour of tubes in a certain time window can be described by four time dependent variables, namely amplitude A, frequency f (or period P), phase φ and trend (base diameter) d. Each can be calculated from the time-evolution of the diameter or the intensity data, but if not stated otherwise the following results are only derived from intensity analysis.

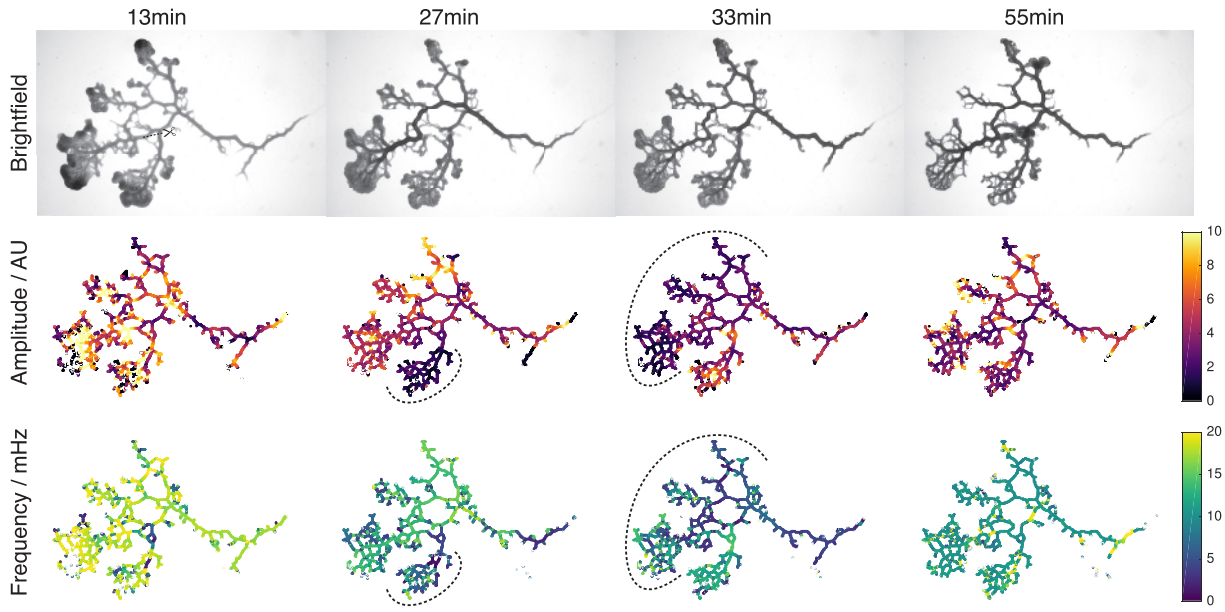

Figure 4. Time evolution of an exemplary network and its spatially mapped oscillation parameters at 13 min, 27 min, 33 min and 55 min. The network was cut in the centre at 17.3 min (*scissor icon*). Top row depicts the raw bright field data, middle row the local amplitude, and bottom row the local frequency. Amplitude and frequency decrease locally, first at the lower sub-network (*small dotted arc*) at 27 min, subsequently at upper sub-network (*large dotted arc*) at 33 min. At 38 min cytoplasmic flows are re-established at the wounding site. Finally, amplitude and frequency values recover.

The trend $d(t)$ is obtained with a moving-average filter with a kernel width of 200 s on each time trace (see figure 3). The dataset is detrended using the calculated trend and smoothed with a Gaussian using a kernel width of 39 s. The kernel widths were chosen to extract the characteristic contraction pattern which usually has a frequency of ∼90 s. The values at every data point are stored as a complex valued time array, with the detrended and smoothed intensity representing the real part and the corresponding Hilbert transform representing the complex part, see S2 for more details. This time array, denoted analytic signal, serves as a basis to get instantaneous phase, frequency and amplitude by computing the angle or absolute value of the complex time series. Finally, the results are mapped back onto the network structure for each time point. In this fashion one can follow oscillatory behaviour resolved in time and space. Furthermore, the maps can be clustered in sub-networks and averaged separately to pinpoint local events in time. It should be mentioned that averaging of results for line plots, i.e. figure 5, is always done after the data-point based analysis took place. In this way for example, the apparent amplitude of the averaged intensity (figure 5(D)) can be lower than the amplitude of each data point averaged (figure 5(B)).

3. Results

3.1. Wounding induces fan growth at cut site

We observe specimens before and after a quick and complete severing of a tube to follow the response of *P. polycephalum* to wounding (see figure 4, movs. S1 and S5). Bright field movies reveal that cutting of main tubes distal to fans triggers cessation of contractions followed by stalling of cytoplasmic flow ($n = 15$ out of 21). After contractions resume the severed tube fuses back together ($n = 21$ out of 21), i.e. flow is re-established, and a fan starts to grow at the cut site. Furthermore, we observe accumulation of body mass close to the cut site which is most prominent in peripheral cuts (figure S2). However, the growth is transient and after a given time the initial morphology is restored and the organism returns to typical behaviour comparable to before wounding.

In consideration of previously mentioned technical limits, we selected one representative dataset with prominent discernible features for network-based analysis. The following findings are derived from this dataset and later compared with other experiments. The specific timing of events in the representative data set is as follows (see figure 4). Two tubes are severed at 17.3 min effectively dividing the network into two parts. In both sub-networks, the size-wise bigger and smaller part, flows stall transiently around 30 min. At 38 min a connecting tube is reinstated and starts to re-establish cytoplasmic flows across the cut site. Until about 63 min a transient fan is created at the cut site. At 90 min the initial morphology is restored and fans are grown elsewhere.

3.2. Spatial mapping reveals localized stalling

We perform network-based analysis on the wounded specimen to extract the interplay of contractions during the healing response. In particular, we map out the amplitude and frequency of contractions spatially (see figure 4, movs. S2 and S3). This allows us to exactly localize the onset of stalling as it goes hand in hand with low values of amplitude and frequency. Likewise, patterns in contraction dynamics in a region of interest are identified by spatially averaging amplitude and frequency in this region (see figure 5).

In the representative dataset, wounding separates the network into two sub-networks. Spatial mapping reveals that