Effective mixing due to oscillatory laminar flow in tubular networks of plasmodial slime moulds

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Abstract

The plasmodium of the unicellular slime mould Physarum polycephalum forms an extended vascular network in which protoplasm is transported through the giant cell due to peristaltic pumping. The flow in the veins is always parabolic and it performs shuttle streaming, i.e., the flow reverses its direction periodically. However, particles suspended in the protoplasm are effectively and rapidly distributed within the cell. To elucidate how an effective mixing can be achieved in such a microfluidic system with Poiseuille flow, we performed micro-particle imaging velocimetry experiments and advected virtual tracers in the determined time-dependent flow fields. Two factors were found to be crucial for effective mixing: (i) flow splitting and flow reversals occurring at junctions of veins and (ii) small delays in the reversals of flows in the veins at a junction. These factors enhance the distribution of fluid volumes and hence promote mixing due to chaotic advection. From the residence time distributions of particles at a junction, it is estimated that about 10% of the volume is effectively redistributed at a junction during one period of the shuttle streaming. We presume that the principles of mixing unravelled in P. polycephalum represent a promising approach to achieve efficient mixing in man-made microfluidic devices.

1. Introduction

The plasmodium of the unicellular, multi-nucleate slime mould Physarum polycephalum may extend over areas of square centimetres or even square metres. Morphologically such a giant cell differentiates into an apical zone, which consists of a poroelastic medium, an intermediate domain, and a vast network of tubular strands or veins (figure 1). Typically, the tubules (veins) have diameters of 10–250 μm [1, 2] and consist of a rather solid ectoplasm and a liquid endoplasm (the protoplasm) that is transported through the veins [3].

In such extended cells, diffusion is no longer an effective mechanism for transportation and distribution of nutrients, oxygen, signalling factors, etc. Therefore, protoplasm is periodically transported back and forth in the tubes, a phenomenon known as shuttle streaming [4]. The driving force for the streaming is an almost periodic [5–7] peristaltic contraction and dilation of the tubes. This peristalsis-induced intracellular transport of protoplasm is also at the basis of the locomotion by creeping of the P. polycephalum cell [8, 9].

In P. polycephalum the protoplasm is an incompressible viscous fluid that is transported in elastic, flexible veins (or strands). This is a similar situation as that encountered in the vascular system [10], except that the oscillatory component of the flow is more prominent in this giant amoeba. In other words, the hydrodynamics found and well-described in the vascular system (as, for instance, outlined by Zamir [10])
can be adapted to the situation in *P. polycephalum*. By contrast to linear veins, in branched hemodynamic flow networks, there are feedbacks between the local pressures generated by the contraction of the veins, the flow velocity of protoplasm, and the compliance of the veins (i.e., the increase in their diameter) [11]. This leads to phase shifts between the flows that emanate from (and lead to) a junction of veins.

Several models have been put forward to describe the interplay between the cellular mechanics of the vein and the peristaltic flow [8, 12, 13]. These models all rely on a coupling between the underlying biochemical (driving) system, that is coupled to cell and fluid mechanics. These models have sparked a novel interest in studying the mechanical properties of *P. polycephalum* [14].

The vein network of *P. polycephalum* forms regular graphs, i.e., graphs with a unique node degree $k = 3$ [1, 2]. The vein segments have different thicknesses and lengths [1, 2] in order to provide for an optimized transport of protoplasm through the cell [15, 16]. With respect to transport efficiency, the architecture of these networks is hierarchic and self-similar [16]. Furthermore, the networks are highly adaptive [1, 2, 15], and an originally dense network may coarsen with time [1, 2, 15–18]. In the absence of any external cue, the contraction of the thicker veins is almost synchronous over the entire network [19], whereas the contraction of narrower strands is phase-shifted. External cues may lead to the spreading of phase waves of vein contractions, for instance, as the cell makes judgments about a cue-induced attraction or repulsion [20, 21].

As the typical diameter of the veins of *P. polycephalum* is around 10–300 μm, the vein system forms a microfluidic system. In the plasmodia, the flow profile of the protoplasm in the veins of *P. polycephalum* is always parabolic, indicating laminar flow [22, 23]. However, particles suspended in the protoplasm are known to be effectively and rapidly distributed within the cell [24], as demonstrated in studies where radioactive traces were introduced in the plasmodium [25], or either mRNA [26], pigments [27], or fluorescent particles [28, 29] were locally injected into a vein of a *P. polycephalum* network. These findings lead to the puzzling question, how an efficient mixing of protoplasm can be achieved in a microfluidic vein network where the flow of protoplasm is always laminar.

Mixing of liquids requires that adjacent volume elements are successively stretched and folded, such that volume elements of different origins, which were originally far apart from each other, may enter in contact with each other. This process has to proceed continuously, so that eventually the contents of the volume elements can homogenize by diffusion at the molecular level. Obviously, such a task can be conveniently achieved in turbulent systems, or in systems where energy is injected to achieve mixing. In microfluidic systems and in systems with laminar flow, mixing becomes a complicated task, and in these cases sophisticated protocols need to be employed to ensure efficient mixing by inducing chaotic advection [30–32].

In the present paper we investigate how protoplasm is efficiently mixed in the vein network of *P. polycephalum*, i.e., in a microfluidic system operating at low Womersley numbers. Therefore, the flow is always laminar, and the flow profiles are always parabolic, i.e., Poiseuillan. To this purpose, we monitor the flow of the protoplasm by micro-particle imaging velocimetry. Subsequently, virtual tracers are started in these flow fields and their trajectories tracked. The fates (final destinations) of the tracers are detected in first-passage time diagrams, in synoptic Lagrangian maps, and their residence time distribution measured. These methods are described in the next section. In section 3 we report on our results, which are discussed in section 4. Here, we elucidate how the protoplasm is efficiently mixed in *P. polycephalum*. Finally, we sum up our findings in the Conclusions (section 5).
2. Materials and methods

2.1. Culture and preparation of plasmodia
Plasmodia of *P. polycephalum* wild-type strain HU195 × HU200 were obtained by germination of sclerotia. To this end, sclerotia were placed on a 1.0% w/v (weight per volume) plain, non-nutritive agar gel (Difco BactoAgar) in a polystyrene box (size: 18 × 25 × 35 cm³) at a constant temperature of 21°C in the dark. The sclerotia germinated and transformed into plasmodia which expanded over the agar matrix. The cell mass was increased by feeding oat flakes (Kölln Flocken) to the plasmodia [33].

An area of about 1 × 4 cm² of the frontal zone of the expanding plasmodium was carefully cut off, and transferred into a round polystyrene Petri dish of 9 cm diameter, which contained 1.0% w/v plain, non-nutritive agar gel. After several hours, a network of tubular strands (veins and venules) developed. A tiny region of this tubular network was observed over time. It contained either a straight, unbranched strand, or a single junction (or branching) of strands, from which three strands emanate. To obtain these geometries of the veins, small lateral venules were removed with a scalpel. The main strand was allowed to heal for at least 15 min prior to the measurement.

2.2. Experimental setup
Experiments were run in a dark room at a room temperature of 21.0 ± 0.5°C, regulated by the air conditioning system of the laboratory. Petri dishes containing the plasmodial networks were placed onto a water-filled plexiglass support and illuminated from below by an array of 10 × 10 green LEDs (520 ± 20 nm). Light of this wavelength did not affect the *P. polycephalum* plasmodia [34]. The light path contained a frosted glass layer to ensure homogeneous illumination of the probe, whereas the water-filled plexiglass support made sure that any heat released by the control circuit of the LED array was absorbed prior to reaching the Petri dish and the probe.

A 2.03 × 1.52 mm² section of the network containing either a single, straight strand or a junction of veins was observed from above through a transmitted light microscope (Wild M3z, Heerburg, Switzerland, magnification *M* = 40×) to which a black-and-white CCD camera (Sony XC-ST70, 640 × 480 px, resolution 3.17 μm px⁻¹) was attached. Images were acquired at a sampling rate of 20 frames s⁻¹ and stored on a computer for later analysis.

2.3. Micro-particle imaging velocimetry
Nuclei, organelles, and starch granules suspended in the protoplasm were used as natural tracers for the cytoplasmic flow in the veins. The illumination intensity was adjusted such that the image of the plasmodium was slightly overexposed, thus allowing for the monitoring of these internal tracers. The time-dependent flow fields in the veins of *P. polycephalum* were obtained by micro-particle imaging velocimetry (μPIV) [35]. This method is based of the bulk illumination of the probe and it measures the average flow field in a shallow liquid layer, the depth of which is determined by the depth of field (focus range) *s* of the optical system. The depth of field

\[
s = \frac{\lambda n}{A^2} + \frac{nε}{MA}
\]

is the depth of the liquid layer that lies in the focus, and it depends on the wavelength *λ* of the illumination, the refractive index *n* of the medium between the object and the lens (i.e., air), the resolution *ε* of the CCD chip, the magnification *M* of the microscope, and the numerical aperture *A* of its objective. In our setup, *λ* = 520 nm, *n* = 1, *M* = 40, and *A* = 0.2409, such that the depth of the fluid layer monitored by μPIV was *s* = 9.29 ± 2.20 μm. Domains and hence fluid flows outside of this window are not accessible by μPIV [35].

The acquired raw images were subjected to background subtraction using a sliding window algorithm. The respective background images were obtained by averaging over the 30 images preceding and the 30 images following the raw image. This procedure yields positive and negative values for the intensity. Next, an offset is added to the background-subtracted images such that they contain exclusively positive grey values. These processed images were used to calculate the flow fields by μPIV.

To reduce the size of the data to be processed, prior to calculating the flow fields, the domains of the images which did not contain the cell were excluded. The flow field displacement vectors at each of the points of the vein were obtained by calculating the cross-correlations between the square interrogation areas (of Δx = 16 px and Δy = 16 px size) and the shifted interrogation areas. The assembly of all displacement vectors yield the flow field at any time point. These flow fields were stored for later analysis.
2.4. Data analysis
The profiles of the flow in straight, unbranched vein segments were measured along a section perpendicular to the veins by μPIV. To this purpose, 79 profiles were acquired along a vein at a spacing of $\Delta y = 25 \mu m$. The flow was measured at a sampling rate of 20 frames s$^{-1}$, and the velocity integrated over 1 s intervals, thus averaging over a total time of 4 s. In addition, the velocities $v(y)$, where $y$ is the position along the diameter of the vein, were averaged over all 79 profiles.

The mean flow velocity $\bar{v}$ of the protoplasm is calculated from the flow field data as

$$\bar{v} = \frac{\sum_{i=1}^{Y} \sum_{n=1}^{N} v_{y} Y_{N}}{YN}$$

(2)

where $y$ is the spatial position along the cross section of the vein of width $Y$ (measured in increments of $\Delta y = 25 \mu m$) and $N$ is the number of subsequent velocity measurements made at $\Delta t = 0.05$ s. The flow field at any point is local, but it contains information about the area covered by the interrogation window, i.e., an area of $16 \times 16$ or $51 \mu m \times 51 \mu m$ (see previous section). For the mean velocity data displayed in section 3.3.2, $N = 10$, yielding a temporal spacing of the mean velocity data of $\Delta t = 0.5$ s.

The dynamics of the flow in the veins were visualized by tracking virtual tracers in the flow field, thus providing information about any possible mixing of the protoplasm. Virtual tracers are point-like, mass-less, and drag-free particles that advect with the flow field. Their trajectories were calculated using the experimentally determined time-dependent flow fields.

To investigate the flow fields and the advection of virtual tracers therein, two initial conditions were used. In the first, an array of either $50 \times 50$ or $60 \times 60$ closely packed virtual tracers is started in the vein, and they are colour-coded according to their starting position. These experiments are used to track the flow of the particles, visualize the mixing in the protoplasm, and to construct first passage time maps. In the second starting condition, virtual particles are started along a line $y$ perpendicular to the vein. Here, $y = 0$ is the inner edge of the vein shown at the bottom part of the vein. At each time step (i.e., at intervals of $\Delta t = 0.05$ s), a new set of virtual particles is started from this line. These data are used to construct synoptic Lagrangian maps and to determine the residence times of fluid volumes in the immediate vicinity of the junction of veins.

The information stored in the trajectory of every particle can be collected in synoptic Lagrangian maps (SLMs) [36, 37]. SLMs are a data presentation (and compression) technique that presents the information on a multitude of trajectories of flow tracers in the form of a map [36]. These maps are synoptic as they provide a general view on the principal parts of the subject. In the present paper, SLMs are geographic maps where each virtual particle is referenced (colour-coded) according to its final position, namely the vein segment through which it leaves the region of interest. This means, that in the SLMs we colour-code the particle according to its fate.

The residence time of each virtual tracer in the region of interest is also plotted in form of a map. Here, the colour-coding of each tracer departing from a position $y$ and time $t$ indicates for how long the tracer remains in the region of interest. This region is delimited by the two or three target lines in the straight and the branched vein segments, respectively.

3. Results

3.1. Laminar flow
Micro-PIV measurements along a line placed perpendicularly to a straight and unbranched vein (of 330 $\mu m$ internal diameter) demonstrate that the flow field developed in the strand is indeed parabolic (figure 2), indicating a laminar Poiseuille flow. Such a flow profile has also been reported in earlier studies [22, 23]. From the measured data, the Womersley and the Reynolds numbers may be calculated.

The Womersley number $\alpha$ describes the ratio of the transient, oscillatory inertial force to the viscous force acting in a system with periodically reversing direction of flow. It is defined as

$$\alpha = \frac{r}{\sqrt{\frac{\omega \rho}{\eta}}}$$

(3)

where $r$ is the characteristic length, i.e., the radius of the tube, which for a wide vein may reach $r \approx 200 \mu m$. In equation (3) $\rho$ is the density of the protoplasm and $\eta$ its dynamic viscosity. The angular frequency $\omega = 2\pi / T$ of oscillations of the peristaltic pumping is about $0.07 \text{ s}^{-1}$ for a typical period of peristaltic oscillations $T$ of 90 s. The density and the dynamic viscosity of the protoplasm of *P. polycephalum* are reported to be $\rho = 1120 \text{ kg m}^{-3}$ [38] and $\eta = 2.0 \times 10^{-3} \text{ Pa s}$ [39–41], respectively. This yields $\alpha \approx 0.04$. Such a small Womersley number means that the frequency of peristaltic pulsations is sufficiently low to
Figure 2. Velocity profile recorded along a line intersecting a strand perpendicularly obtained by collecting 79 individual flow profiles at 0.05 s time intervals. These profiles were collected around the maximum flow velocity. The local flow velocities were measured in spatial increments of $\Delta y = 25 \, \mu m$. A parabolic profile is fitted to the velocity data points (symbols) and the error bars represent the standard deviation of the 79 local flow profiles. The internal diameter of the vein is $\approx 330 \, \mu m$, ranging from $y \approx 120 \, \mu m$ to $y \approx 450 \, \mu m$. The ectoplasm (cell walls) is located at $y \leq 120 \, \mu m$ and $y \geq 450 \, \mu m$, respectively. As the vein is subjected to peristaltic contraction and dilation, the positions in the vicinity of the borders (i.e., $120 \leq y \leq 150 \, \mu m$ and $400 \leq y \leq 450 \, \mu m$) show some deviation from the parabolic velocity profile.

Figure 3. Snapshots of a propagating array of $50 \times 50$ virtual tracers. The left half of tracers are labelled in red, the right half in blue. As the tracers are advected, the array gets slightly dispersed. Initially, the tracers propagate to the left; later, the flow inverts its direction (this occurs between the last panel of the middle row and the first panel of the bottom row). The snapshots are taken at time intervals of 0.5 s. Dimensions: $2.03 \times 1.52 \, mm^2$. A video of the advecting particles is provided as supplementary material (video V1).

allow the velocity profile to remain parabolic at all times [42]. Furthermore, the flow profile is in phase with the instantaneous value of the pressure gradient driving the flow [42].

We have also calculated the Reynolds number $Re$ of the protoplasm of *P. polypephalum*. It is defined as the ratio of the inertial forces to the viscous forces that are active in a fluid,

$$ Re = \frac{2 rv \rho}{\eta}, $$

where $v$ its velocity of the protoplasm. We have estimated the oscillatory (i.e., maximum) Reynolds number $Re_{\text{max}}$ [43] attained during protoplasmic flow in the strands of *P. polypephalum* using data directly available from our experiments and from literature. The maximum Reynolds number is obtained using the maximum velocity attained during shuttle streaming, which in our experiments was $1.20 \, \text{mm s}^{-1}$ (figure 2),
and in experiments by Kamiya and by Bykov et al was 1.3 mm s$^{-1}$ [4] and 1.5 mm s$^{-1}$ [22], respectively. Using $v = v_{\text{max}} = 1.20$ mm s$^{-1}$ as well as $r= 0.20$ mm as the typical diameter of a larger vein of $P. \text{polycephalum}$, we obtain an oscillatory Reynolds number for the flow of protoplasm in a vein of $Re_{\text{max}} \approx 0.27$. This indicates that in the protoplasm viscous forces slightly dominate over the inertial forces, however, inertial forces cannot be neglected during pulsatile flow specially in broader veins. When the protoplasm flows at a slower pace, the role of the inertial forces becomes even smaller. Most importantly, however, is the fact that the maximum Reynolds number attained in the protoplasm is four orders of magnitude lower than that required for the onset of turbulence in a cylindrical tube. Hence, the flow in the protoplasm remains always laminar. Indeed, in our $\mu$PIV measurements, we never observed any occurrence of turbulence.

3.2. Flow in a straight unbranched strand

The dispersion of particles is first studied in a long, straight, unbranched vein segment. To this purpose, an array of $50 \times 50$ virtual tracers is started at the right side of the vein (figure 3 and video V1 in the supplementary material). As the packet of virtual tracers is advected to the left, the tracers get dispersed. However, as the flow rate is inverted, the particles are advected to the right, towards their initial position, and they get slightly compacted again.

This observation is corroborated by the first passage times of the virtual tracers across a line close to the starting position of the tracers (figure 4(a)). The arrays of tracers advecting to the left show square shaped distributions, and the tracers labelled in red precede those in blue (figure 4(b)). When the particles return, the particles labelled in blue arrive prior to those coloured in red. It is worth to note that there is no reversal of the geometric arrangement of the red and blue tracers in the flow field. The returning particles remain densely packed, however, their distributions adopt a more Gaussian shape.

3.3. Flow at a junction of veins

3.3.1. Flow fields and first passage times

The situation becomes more complex at the junctions that always connect three veins. The inversion of the direction of flow of the protoplasm does not set in simultaneously in the three veins forming the junction.
Figure 5. Flow field at a junction of veins during a flow reversal from the left to the right. The flow fields were determined by μPIV. (a) Flow from left to right (at time $t = 0$). (b) Stagnant protoplasm in the top left vein ($t = 0.85$ s). (c) Flow reversal at the top left vein ($t = 1.05$ s). (d) Stagnant protoplasm in the right branch ($t = 1.25$ s). (e) Stagnant protoplasm in the bottom left branch ($t = 1.40$ s), and (f) flow from right to the left ($t = 2.15$ s). Dimensions: $2.34 \times 1.75$ mm$^2$. The corresponding video is provided in the supplementary material (video V2).

Depending on the local pressure differences between each of the three veins connected to the branching point, the flow inversion in the individual vein segment may take place at slightly different times. This is illustrated by the μPIV flow fields in figure 5 and in video V2 (supplementary material). Here, we observe that the protoplasm initially flows from the two veins at the left into the segment at the right, from where it leaves the region of interest (figure 5(a)). Eventually, at the end of the process, the direction of flow is inverted and the protoplasm flows from the right vein into the left veins (figure 5(f)). The inversion does not take place instantaneously. Instead, we first observe that the flow in the top left branch ceases (figure 5(b)), which after a short delay is replaced by a flow pattern where the protoplasm entering through the bottom left branch leaves the region of interest through the two other veins (the top left and the right; figure 5(c)). Shortly thereafter, the flow in the right vein in inverted (figure 5(d)), thus leading to a flow from both, the right and the bottom left vein into the top left vein. Finally, the flow in the bottom left branch changes its direction (figure 5(e)). This leads to a flow of protoplasm from the left branch to the two branches at the left. This situation corresponds to the full inversion of the flow pattern, which then persists for almost half a period of the shuttle streaming.

The small delays during the inversion of the flow direction in the individual veins attached to a junction open up the possibility of an efficient mixing of the protoplasm. This is visualized by tracking the trajectories of an array of virtual tracers during an episode of flow reversal. The evolution of the array of virtual tracers in these veins is depicted in figure 6 and in video V3 (supplementary material).
Figure 6. Snapshots of a propagating array of $60 \times 60$ virtual tracers. The left half of tracers are labelled in red, the right half in blue. As the tracers are advected towards the left, they enter the junction where the array of tracers splits and the virtual tracers are advected into the two left branches of the vein network. Whereas the virtual tracers transported into the lower left branch flow out of the region of interest, the tracers that flow to the top left branch are slowed down and subsequently revert their direction of flow. These particles are then advected to the lower left branch, where they experience another flow reversal. Finally, these particles are transported back to the right branch, where they leave the region of interest. The snapshots are taken at time intervals of $0.7 \text{s}$. Dimensions: $2.03 \times 1.52 \text{mm}^2$. A video of the advecting particles is provided in the supplementary material (video V3).

When a fluid volume (and the virtual tracers advected with it) reaches the branching of veins, it splits and portions of the fluid are transported into the two branches. This also leads to a spitting of the array of virtual tracers (figures 6(b) and (c)) and consequently to a different fate of the tracers. In figure 6 the tracers transported through the bottom left vein are carried out of the observation area (figure 6(e)). By contrast, the tracers that have entered the upper left branch experience a reversal of the direction of flow (figures 6(d)–(f)) and return to the branching point. There, they are taken up by the flow and transported into the bottom left vein (figures 6(g)–(i)). However, here, too, the direction of flow is inverted, so that the particles are transported back to the right vein, where they had originally started from (figure 6(l)). Eventually, they leave the region of interest through this right branch. As a consequence from the slight asynchronicity in the flow reversal, the virtual tracers that originally started as a dense array may suffer different fates and reach different destinations.

The reversal of the direction of flow in one vein at a junction and the subsequent injection of the fluid volumes into the flow that still connects the other two branches (where the flow has not yet reversed its direction) also leads to an inversion of the sequential ordering of the virtual tracers advected with the fluid. This can be seen in figure 6, where the red tracers originally preceded the blue particles (figures 6(a) and (b)). After flow reversal in the top left vein and injection into the bottom left segment, the sequential ordering of the virtual tracers was inverted with the blue tracers preceding the red ones (figures 6(g)–(i)). This inversion of the sequential ordering is also evident in the first passage times of the particles at the intersecting line in the bottom left vein. Here the packages of tracers arriving directly (at $1.7 \leq t \leq 2.3 \text{s}$) have the opposite sequential ordering as those arriving after the flow reversal (at $4.5 \leq t \leq 5.4 \text{s}$) (figure 7).

The first passage time distribution in figure 7 also shows that the virtual particles arrive at the bottom left vein at distinctively different times. Those tracers advected directly to this branch, arrive there at $1.8 \leq t \leq 2.3 \text{s}$, whereas those subjected to the reversal of the flow direction in the top left vein arrive at the intersection line at $4.6 \leq t \leq 5.3 \text{s}$. Hence, there is a considerable delay between the two groups of virtual
tracers that were started as a geometrically tight array. This means that the packages of virtual tracers were separated by a substantial volume of protoplasm of different origin than the volume elements containing the tracers. Furthermore, the distributions of the tracers arriving later at the position for the measurement of the first passage time are broader and less Gaussian-shaped than those for the tracers arriving directly. Taken together, these observations, i.e., (i) the considerable distortion of the originally tightly packed array of tracers (figure 6), (ii) the substantial broadening of the first passage time distribution (figure 7), and (iii) the distinctly different arrival times of the different packets of tracers at the position of the measuring line (figure 7), provide substantial evidence for the stretching and folding of fluid elements at a junction of veins that do not reverse the direction of the protoplasmic flow synchronously. Thus, substantial mixing of the protoplasm can be achieved at branching points in the vein network of *P. polycephalum*.

### 3.3.2. Synoptic Lagrangian Maps

So far, we have studied the dynamics of an array of virtual tracers advected at a junction of veins. The virtual tracers were started immediately before the asynchronous reversal of the direction of flow set in in the veins. In order to monitor the dynamics of flow and mixing of protoplasm at a junction of the vein network over a couple of periods of the shuttle streaming, we construct SLMs of the destination of the tracers. At each time step, virtual tracers were started along a line perpendicular to the vein as indicated in figure 8. In addition to SLMs, we also monitor the residence time of the virtual tracers and the mean
Figure 9. (a) Mean flow velocity in the top left (‘red’) vein segment of figure 8. Positive values of the velocity indicate that the protoplasm flows towards the top left (toward the red line), whereas negative velocities indicate that the protoplasm flows towards the junction (figure 8). (b) Synoptic Lagrangian map (SLM) and (c) map of the residence times of the particles in the domain delimited by the green, red, and blue lines of figure 8. At each time step (i.e., each 50 ms), 70 virtual tracers equidistantly spaced along the starting line (at intervals of $\Delta y = 4.0 \mu m$) are started. Their fate is colour-coded in the SLM, indicating through which of the three veins a particle leaves the region of interest. The time series are continued in figure 10.

The mean flow velocity $\bar{v}$ (equation (2)) of protoplasm in the top left branch of the network shown in figure 8 oscillates in time due to the shuttle streaming. The flow direction is inverted periodically, roughly every 100–120 s (figures 9 and 10), demonstrating that there is a certain variation in the period of the shuttle streaming as well as in the amplitude of the mean flow velocity. When $\bar{v} > 0$, the volume elements were transported into the upper left vein and they left the network though this vein. By contrast, at velocities $\bar{v} < 0$ fluid is transported towards the bottom left and/or right veins. This is also evident from the
Figure 10. Continuation of the time-series shown in figure 9.

SLM of the destination of the virtual tracers, i.e., whenever $\bar{v} > 0$ the particles leave though the ‘red’ vein (figures 9 and 10).

An interesting behaviour is observed when the flow inverts, as this causes the virtual tracers to leave the vein network though another vein segment. This is, the particles do no longer leave the network through the ‘red’ channel but though the ‘green’ and/or ‘blue’ vein. The SLM, however, shows that as $\bar{v}$ changes sign, the identity of the outlet channel is not uniquely determined: For instance, at $t = 170$ s and 300 s, all particles flow into the right (‘green’) vein, whereas at $t = 680$ s and 800 s all particles leave the network through the bottom left (‘blue’) vein (figures 9 and 10). In addition, situations where flow inversion leads to a simultaneous flow into the right (‘green’) and bottom left (‘blue’) veins are also observed, e.g., at $t = 425$ s (figures 9 and 10). During the episodes where $\bar{v} < 0$ the vein through which the tracers leave the region of interest may or may not change, e.g., for $290 < t < 360$ s, where the particles leave exclusively through the
Figure 11. Synoptic Lagrangian maps obtained for starting positions located at the different branches emanating from a junction. Top: coordinates of the starting vectors for the synoptic Lagrangian maps (yellow lines): the three starting vectors are located close to the junction in the three branches leading to the ‘green’, ‘blue’, and ‘red’ exits. Accordingly, the latter are labelled ‘green’, ‘blue’, and ‘red’ branches. Bottom: synoptic Lagrangian maps of the destinations of the virtual tracers that start at the three branches emanating from the junction. At each time step (i.e., each 50 ms), 70 virtual tracers equidistantly spaced along the starting line (at intervals of $\Delta y = 4.0 \, \mu m$) are started.
Figure 12. Correlation between the residence times $\tau$ of virtual tracers in the neighbourhood of a junction and the number of flow reversals experienced by the virtual tracers. (a) Residence time distribution of the virtual tracers flowing through a junction without flow reversal (blue), and (b) of virtual tracers that experience flow reversals once (yellow) or twice (red) at the junction. Notice the different scaling of the relative abundance in (a) and (b). The binning interval was $\Delta t = 0.1$ s.

right (‘green’) vein, or at $800 < t < 870 \text{ s}$, where they leave the junction initially through the bottom left (‘blue’) vein, but later through the one at the right (‘green’ vein) (figures 9 and 10).

The SLM of the virtual tracers (figures 9 and 10) shows that the destinations of the fluid elements change from cycle to cycle of the (almost periodic) protoplasmic shuttle streaming. In fact, for tracers flowing into the junction, there is no repetitive pattern for their destinations. This leads to a macroscopic mixing of fluid particles occurring in time intervals of $10–60 \text{ s}$, that provides for an almost aleatory distribution, and hence macroscopic mixing, of the protoplasm.

The appearance of the SLM depends on both, the position of the initial vector of virtual tracers and the position of the limits of the region of interest (as, for instance, defined in figure 8). This dependence is illustrated in figure 11, where for a given junction and flow field, the propagation of the initial tracers was started at different positions. Each of the 3 starting vectors laid close to the junction, but on different veins. The SLMs obtained using the same flow field data but different starting vectors for the propagation of virtual tracers differ. It is observed that the fraction of tracers leaving the region of interest through the vein where the tracers were initiated is favoured in the SLM constructed at an intersection on the same vein (figure 11). Despite of the dependence of the appearance of the SLMs on the geometric positions of the initial vector and the location where the SLM is constructed, the main features can be found in all of the SLMs, namely flow reversals and flow splittings. In addition, it is also evident that the redirection of flows to the branches emanating from a junction did not follow a regular repetitive pattern (figures 9–11).

3.3.3. Residence time distributions

The maps of residence times of the virtual tracers in the region of interest around a junction in figures 9 and 10 reveal that the big majority of fluid elements (or virtual tracers) has a very short residence time of $\tau < 2.5 \text{ s}$. These fluid elements are just transported through the junction. However, there are also episodes where the virtual tracers remain significantly longer in the veins immediately adjacent to the junction.
Figure 13. Residence time distribution of 30,000 virtual tracers in the region of interest of a junction of veins. The binning interval was $\Delta t = 0.1$ s. The mean residence time $\tau_m = 1.87$ s, and the standard deviation $\sigma = 0.49$ s. The fraction of virtual tracers that have a long residence $\tau > \tau_m + 0.5\sigma = 2.12$ s in the region of interest is represented in blue. This is the case for $\approx 10\%$ of the virtual tracers.

When investigating the SLM of the particle destinations and the residence time maps (figures 9 and 10), it becomes apparent that the episodes where tracers have longer residence times correspond to events either of flow inversion in a vein or of flow splitting between two veins (as also seen from the first passage times in figure 7). These events of flow inversions in individual branches lead to a mixing process on a more microscopic scale, and they involve much less virtual tracers and occur on a shorter time scale (of up to 2–8 s).

The importance of flow reversals for the mixing of fluid volume elements can be assessed from the residence time $\tau$ of virtual tracers in the vicinity of a junction. In fact, the residence times $\tau$ and the rate of flow reversals that a virtual tracer has experienced are correlated, as seen from figure 12. The residence time distribution also shows that the probability of undergoing a flow reversal is much lower than that of flowing straight through the junction, and it increases with the rate of reversals (figure 12).

When assembling the residence times $\tau$ of all virtual tracers advected in figures 9 and 10 into a single histogram, they show a distribution with a heavy tail for large values of $\tau$ (figure 13). The mean value of the distribution is $\tau_m = 1.87$ s and its standard deviation $\sigma = 0.49$ s. Residence times shorter than 1.3 s were not observed. We consider all virtual particles that remain in the region of interest for times $\tau \leq \tau_m + 0.5\sigma = 2.12$ s as particles that simply flow through the junction with the laminar flow. By contrast, tracer particles with residence times longer than $\tau > \tau_m + 0.5\sigma$ are considered as particles that are subjected to mixing processes due to either flow reversal (cf figure 12(b)) or flow splitting. The abundance of particles with such long residence times is $\approx 10\%$ (figure 13), allowing for the assessment of the efficiency of mixing due to flow reversals at a junction of veins.

4. Discussion

We have investigated how efficient mixing arises in the vein network of *P. polycephalum*. This network is a microfluidic system, where the driving force for the periodically changing flow is provided by peristalsis. The flow is always laminar, as observed in our $\mu$PIV measurements (figure 2), by Doppler optical coherence tomography [22], and particle tracking velocimetry [23]. The Womersley and maximum Reynolds numbers calculated using the values of the physical parameters measured in or known for *P. polycephalum* are $\alpha \approx 0.04$ and $Re_{max} \approx 0.27$, in good agreement with values obtained in narrower veins of *P. polycephalum* [24, 44]. The low Womersley and oscillatory Reynolds numbers corroborate that the flow is laminar at all times. Hence, the question addressed in the present paper is how efficient mixing is achieved in a microfluidic system operating exclusively under laminar flow.

The tubular network of *P. polycephalum* consists of unbranched segments and of junctions of veins. In the following, we discuss the contributions of these two elements to the mixing of the protoplasm in
**P. polycephalum.** In straight, unbranched vein segments mixing is provided mostly by molecular diffusion, since in straight tubes the protoplasm is shuttled back and forth, the net contribution of unbranched tubular segments to the mixing of protoplasm is small. By contrast, the junctions of veins play a crucial role for enhancing the mixing of protoplasm in the cell. The contribution of vein junctions relies on two features, namely the change in geometry provided by the junctions and the fact that the shuttle streaming is not strictly periodic. In fact, it is the interplay of these two factors that provides for most of the enhancement of mixing in the tubular vein system of *P. polycephalum*.

At a junction, the pattern of flow distribution is altered. When protoplasm is delivered to the vein junction through two veins and transported away from it through the third one, volume elements of protoplasm of different origins are brought into contact, increasing the contact area between protoplasm of different origins, thus promoting the diffusional mixing of protoplasm. Conversely, the junction may act as an obstacle that splits the liquid volume entering from one vein, thus distributing the liquid among the two exiting veins. In this case, some of the originally adjacent volume elements are separated and may have a different fate. Since the shuttle streaming is almost—but not exactly—periodic, the volume elements do not return to their initial positions after a period of the shuttle streaming.

The fact that the shuttle streaming is not strictly periodic, but that it presents fluctuations around its mean frequency provides for another crucial contribution that drastically enhances the efficiency of the mixing of protoplasm in *P. polycephalum*. The slight aperiodicity of shuttle streaming is associated to variations of the pressure differences between the three veins emanating from a branching point. These pressure differences determine the patterns of protoplasmic flow at a vein junction. As seen from the synoptic Lagrangian maps in figures 9–11 the fluctuations in periodicity of the shuttle streaming lead to complex, not necessarily repeating patterns of protoplasmic flow at a junction. For instance, the temporal sequence of veins through which a volume element leaves a junction does not change in a repetitive fashion (figures 9–11). This leads to an aleatory redistribution of volume elements in the vein network.

The main effect of the fluctuations in the shuttle streaming consists in changing the flow pattern through the veins at a junction. Because the reversals of flow direction in the individual veins do not take place simultaneously, situations occur where the flow in two veins retains its direction while the direction of flow in the third branch switches (e.g. in figure 6). In such a case, volume elements that were transported in the branch with reverting flow are advected back to the junction and eventually leave through the vein where another part of the originally adjacent volume elements have left the junction. Although the volume elements eventually leave through the same vein, they do so at pronouncedly different times (as evidenced by the first passage times in figure 7 and in the maps of residence times, see figures 9(c) and 10(c)). Hence, the excursions into a vein with flow reversal is a very effective way to rip originally adjacent volume elements apart from each other and to put them into a new context. Note that this reshuffling of volume elements is exactly what is required to enhance mixing.

Flow reversals in a vein occur once a period of the shuttle streaming, i.e., once in about 90–100 s. Although the abundance of flow reversals is relatively low, they constitute the most important local element leading to the enhanced mixing at a junction of veins. Nevertheless, it is estimated that during one period of the shuttle streaming, about 10% of the volume elements are subjected to mixing by flow reversal (figure 13). Given that during one period of the shuttle streaming a volume element in average passes through 6 vein junctions, the reshuffling of volume elements may provide for a fairly efficient mechanism for the mixing of protoplasm.

From the biological point of view, the mixing mechanism described above combines an efficient use of energy to achieve efficient mixing, especially, since no energy needs to be dissipated to create any turbulent eddies. The vein network of *P. polycephalum* is a natural microfluidic system. The mechanism of mixing taking place in its tubular network represents an approach which is very powerful in both the mixing efficiency and the low amount of energy dissipated to achieve the mixing. To our knowledge, such an approach has so far not been realized in man-made microfluidic systems. We conjecture that, at a time where microfluidics is intensively sought-after, the implementation of the principles of mixing effective in *P. polycephalum* may represent an alternative and very efficient solution for achieving mixing in engineered microfluidic devices.

5. Conclusions

We have investigated the apparent paradox that protoplasmic flow in the veins of *P. polycephalum* is always laminar and that mixing of protoplasm throughout the giant cell is efficient and rapid. We found that the paramount contribution to effective mixing is provided by the flow pattern at the junction of veins. Here, flow reversals and specially the splitting of the flow between two of the three veins emanating from the junction in conjunction with slight delays at the onset of flow reversals in these three veins cause efficient
mixing. Furthermore, these two factors also create a pattern where protoplasmic flow is distributed among the veins in a highly variable manner. We estimate that $\approx 10\%$ of the liquid volume is efficiently mixed at each junction per oscillatory period of the shuttle streaming. We believe that these principles that lead to efficient chaotic advection in *P. polycephalum* may also be very useful for providing efficient mixing in man-made microfluidic devices.

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