Running title: Blood flow in a developing tunicate

Title: A quantitative study of blood circulation in the developing adult ascidian tunicate *Ciona savignyi* (Cionidae).

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Abstract

Development of the adult ascidian tunicate starts when the tadpole larvae attaches to a surface. In approximately two days the solid tadpole will metamorphose into two joined concentric hollow cylinders. The outer cylinder is the body and the inner cylinder is the branchial basket containing openings (stigmata) lined with cilia that pump water through a mucus net that traps food. In six days a heart and circulatory system has formed and blood is pumped through the branchial basket and a smaller visceral cavity containing the heart, stomach, intestine, and gonads. At this stage the animal is quite transparent and moving blood cells are easily distinguished from the fixed cell network of the animal body. The human eye-brain is good at identifying moving cells, but the area of high resolution is limited as is the ability to remember multiple events. However, sequential video frames obtained using a consumer grade camera mounted on a low power microscope, contains the information needed to identify and document moving cells using free open source software described in this report. Subtraction of sequential frames results in a blank difference image if the frames are the same, but produces positive-negative image pairs of cells that have moved during the frame interval. The collection of many sequential difference images thus produces a map of the circulatory system. At six days the circulatory system consists of two perpendicular loops. The larger longitudinal (sagittal) loop runs from the heart along the ventral edge of the branchial basket to a loop around the oral siphon, then back along the dorsal edge of the basket, through three branches in the small visceral cavity, and returns to the heart. One or more transverse loop(s) transports blood from the ventral to the dorsal vessel across the sides of the branchial basket and around the stigmata. Blood cells traverse the longitudinal loop in about 11 sec. As the tunicate matures the number of stigmata increases and the transverse loops develop branches. The branch points then migrate to the dorsal and ventral vessels to form a

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series of parallel transverse vessels. In the brachial basket blood cells move in both transverse and longitudinal direction around the stigmata.

Introduction

Tunicates are believed to be the sister group to vertebrates (Delsuc et al. 2006), and thus a comparison of the two subphyla, especially their early development, could suggest the process of evolution from a common ancestor. The model tunicate *Ciona intestinalis* has been used in the majority of embryological studies which have focused on development of the larval tunicate up to its metamorphosis into the adult form (Stolfi and Christiaen 2012). Cellular precursors of the heart have been mapped in the larval tadpole (Christiaen et al. 2009; Davidson 2007; Davidson et al. 2006; Stolfi et al. 2010), but a beating heart and functional circulatory system does not form until after metamorphosis.

The tunicate studied in the present report, *Ciona savigny*, is morphologically almost identical to *C. intestinalis*, and Milar (1953) considered them to be the same species. However, the genus *Ciona* is very polymorphic, and the genomic sequences of these two species have significant differences (Berna & Alvarez-Valin 2014; Berna et al. 2009).

Metamorphosis

Growth and development of ascidian tunicates occurs in two stages. In the first stage an egg fertilized in the water column develops into a larva of about 2000 cells which is released from the egg as a free swimming “tadpole”. Within one or two days the tadpole attaches to a solid surface which initiates the second stage, a dramatic metamorphosis followed by growth and development to produce the adult sessile animal(Chiba et al. 2004; Karaiskou et al. 2015; Passamaneck & Di Gregorio 2005).

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As mentioned before, the first stage has been the focus of most studies of *Ciona* development, since it is in this stage that the notochord and muscular tail is formed, which identifies the animal as a chordate. However, it is only in the second stage of development that a functional circulatory system, the subject of this report, develops. Gene expression studies in this second stage are now appearing(Azumi et al. 2007).

Open and closed circulation

In tunicates the direction of blood flow reverses every one to four minutes which would be compatible with blood flowing back and forth between two large sinuses. However, the large volume of blood pumped in each direction is only compatible with circulation Krieble (1968).

In the literature circulation in tunicates is usually described as "open" (Davidson 2007; Monniot et al. 1991; Passamaneck & Di Gregorio 2005; Satoh 1994; Satoh 2016), a system typical in crustaceans and insects. Blood is pumped by the heart into a short vascular tree, exits and flows in direct contact with cells and collects in a large, pericardial sinus. No such sinus has been described in *Ciona*.

Circulation in tunicates is sometimes claimed to be open because the blood vessels lack endothelial cells, which line the internal walls of vertebrate blood vessels and prevent plasma from flowing freely into the interstitial space. Citations are not given, but the source is probably the comprehensive 123 page monograph *Ciona*, by Millar (1953), in which 20 pages are devoted to the circulatory system. In this publication reference to endothelial cells lining blood vessels consists of one sentence stating they were only found associated with vessels near the heart. There is no mention of criteria used to define endothelial cells, or which vessels in the animal were studied. A recent study of the ascidian tunicate *Corella inflata* described a circulatory

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system that retained high molecular weight dextran to the same degree seen in mammalian vessels (Konrad 2016). Thus in this tunicate at least, circulation appears to be closed.

Blood vessels and organs

The stereotypical image of the circulatory system in vertebrates is a network of long branched tubular blood vessels connecting distant discrete organs. However, blood flows through individual organs in a vascular architecture characteristic of the function of that organ (Augustin & Koh 2017), e.g. in the liver blood percolates around cells in an amorphous sinus network.

The branchial basket

The branchial (gill) basket in tunicates in general, and particularly in the young tunicates studied in this report, represents a large fraction of the volume of the animal and have a striking symmetry. Thus any visual study of circulation is likely to emphasize the circulation in the branchial basket.

Visualizing circulation

The adult *Ciona* tunicate is large enough that it is possible to inject dyed latex into vessels and produce a cast of the circulatory system in the dead animal (Millar 1953). However, this technique becomes difficult for younger and smaller stages and it does not give information about blood speeds (flow) in the living animal. Fluorescent dye can be injected into vessels of living tunicates, e.g. *C. inflata*, to produce dramatic images of the vessel network (Konrad 2016). However, injection becomes difficult in small, young animals, and it also provides no information on blood speeds in the circulatory system.

The young tunicate for 5-20 of days after attachment to a solid substrate has a simple circulatory system and is very transparent. The flow of moving blood cells can be easily seen by the human

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eye-brain system, but the field of acute view is small and humans can only remember a very limited number of cell movements over a short time since there is no mechanism to archive details of multiple visual images.

However, blood circulation is easily recorded as video, a sequence of images typically acquired 30 times a second. Moving objects can be selectively seen in each image by subtracting the previous image, with subtraction done pixel by pixel across the x-y plane. The difference between two images is essentially the definition of movement, change with time.

There is an extensive literature on the characterization of particle flow, reviewed by Willert (1991), using different techniques appropriate to the flow and embedded in the advancements in optical and electronic technology over the last several decades. Techniques used by Schwerte et al. (2000; 2003) for following the blood distribution in zebrafish may be the closest to those used in this study. They used a video camera sending 30 interlaced frames per second to an analog video tape recorder, and the images were then digitized by a frame grabber computer card. This technology has been replaced in the present study 15 years later by a consumer grade digital camera which produces and stores up to 30 minutes of progressively scanned, i.e. not interlaced, full digital images. The details of algorithms used by Schwerte et al. are somewhat obscured by their use of proprietary software, but as in the present study, one step was the subtraction of images of sequential frames to produce what they termed “shifting vectors”. Since sequential frames of interlaced images are not really pairs of sequential images, the results are not exactly the same as those obtained in the present report. However, they were able to obtain good images of the vascular system for the entire zebrafish and measure cell velocities.

The concentration of blood cells in the blood of tunicates is low compared to most vertebrates, and individual cells are thus typically separated by a distance larger than their movement during

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the 1/30 th second between image frames. Thus it is usually easy to identify images of the same blood cell in sequential frames. However, identification of sequential images of a cell is subject to statistics, and errors can occur in concentrated patches of cells. In addition, even in young tunicates there are opaque structures, so the image of a blood cell can be occasionally obscured.

The results of this report show that a simple, robust, closed circulation of blood cells is established in tunicates by six days after attachment. As the animal develops during the next 30 days more vessels form, and the pattern becomes more complex.

Vessel nomenclature

Blood in tunicates has sometimes been described as moving through sinuses. Spaces containing blood in the tunicate are often quite different from the archetypical tubular vessels that connect the heart to organs in vertebrates. However, as shown in this report, and consistent with observations in the tunicate *C. inflata*, there is a constant blood flow throughout the animal and there are no pools of slow moving blood as would be implied by the term sinus. Thus, the terms vessel, tube, channel or duct would seem more appropriate to describe the circulatory system of tunicates than sinus.

Circulation implies local directional flow. In vertebrates, e.g. fish, blood flows in only one direction, and the term artery or vein in the name of a blood vessel indicates that blood flows away from, or toward the heart respectively. In addition, the structure of the vessel wall is characteristic of flow direction, with arterial walls being thicker and containing more muscle, while venous walls are relatively thin. However, in tunicates blood flows for periods of several minutes in one direction and then reverses direction for approximately the same time. There is essentially no published information on the histology of tunicate blood vessels, but the symmetry (Cirino & Brown 2014)of blood flow would suggest that vessel structure would also be

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149 symmetrical, and vessels in tunicates are not described as arteries or veins. However, it is useful
150 to have a label for a reference flow direction, one that would be consistent for the entire animal
151 and preferably have a biological connotation. In this report flow from heart into the branchial
152 basket (gill basket) will be described as vertebrate flow (V), while flow in the other direction will
153 be described as contra-vertebrate flow (CV). If no directional label is used, vertebrate flow will
154 be implied.

155 Methods

156 Production, care and observation of young tunicates

157 Adult *Ciona savigny* HERDMAN, 1882 were collected in a marina in Sausalito (San Francisco
158 Bay) from the side of a floating dock at a depth of 0.1 – 0.3 m. Adults were kept in a seawater
159 aquarium at temperatures of 16-20C with aeration, and used within 72 hours of collection.
160 Collection of eggs, sperm and fertilization were accomplished essentially by using the
161 ASSEMBLE protocol (Cirino & Brown 2014). Briefly, animals were laid on a 5 mm thick gel of
162 silicon (Slygard 184, Global-Industrial Corp) in a 7 x 7 cm acrylic box and immobilized with 3
163 to 5 pins. An incision was made through the test to expose egg and sperm ducts. Excess water
164 around the sperm duct was removed, the duct cut, and sperm removed carefully with a Pasteur
165 pipet. Immediately before use sperm was diluted in 5 mL of seawater (SW). The eggs are easily
166 collected since the duct is large and individual eggs can be seen. Eggs were diluted in seawater
167 (SW), with 100-1000 eggs in 10 mL of SW in a 60 mm plastic Petri dish. A 0.1 mL aliquot of the
168 sperm suspension was added to each dish of eggs. After 24 hours tadpoles were distributed into
169 multiple 60 mm dishes, with about 20 tadpoles in 10 mL of SW per dish. After an additional 24
170 hours to allow attachment of tadpoles to the bottom, the SW was decanted and replaced with 10

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mL of fresh SW, thus there is an uncertainty of 12 hours in the exact time of attachment. Dishes with 1-3 attached tunicates were used for observation, and SW was replaced daily.

Young tunicates tend to float vertically from the plastic surface on a flexible stalk, and it can be difficult to obtain lateral views. However, small (4 x 4 mm) rectangles are easily cut from plastic coverslips, and when laid over a tunicate force it into a horizontal position. The low density of the plastic results in a small force applied to the animal, but it may still be useful to bend over one edge of the rectangle by 90 degrees to prop up that edge when the plastic is placed over the animal. This produces a space under the plastic square with a triangular cross section to confine the tunicate, and thus translation of the square will bend the tunicate to the desired extent. Tunicates that were confined in this way were observed only one time, even though they typically did not appear to be injured by the procedure.

Staining with neutral red

Attached tunicates were stained by addition of 10 mL of a 0.1% solution of neutral red dye (Cynmar Corp.) in SW for 10 minutes. They were then washed four times in 10 mL of SW.

Image capture and processing

Two optical systems were used to obtain single images and video sequences:

1- a Meiji stereoscopic microscope with a Canon Rebel T3i camera,

2- an Olympus SZX16 stereoscopic microscope with a Canon EOS 6D camera.

Single still images used for documentation were processed using Photoshop (Adobe Systems, Inc.) with adjustments applied uniformly to the entire frame. Video files were converted to image sequences using QuickTime Player 7 (Apple Inc.), and were not modified further before analysis.

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In several cases the paths of moving blood cells in an image sequence was determined manually using Photoshop. The default configuration of Photoshop gives x,y positions to 0.1 pixel, which is useful when one wants to specify the center of an object of 3-16 pixels wide. In this report speed is defined as distance/time, where time is typically 1/30 second, the time between each video frame. The term velocity is reserved, as is convention, for the vector which specifies both speed and direction.

Software

Software to produce and analyze sequential image pair differences was written in Java and implemented as Plug-Ins for the ImageJ open-source application maintained and distributed by NIH (Broeke et al. 2015; NIH 2017). The source code of the two plugins used to produce the images and data reported here are deposited at www.github.com in the 4atunicate/ImagePair repository. The open-source Integrated Development Environment (IDE) Eclipse (Foundation 2017; Vogel 2013) was used in writing Java source code. Java can have a steep learning curve, but the ImageJ platform provides such a powerful collection of supporting functions so a plugin with less than 20 lines of code can reveal the heartbeat of a tunicate.

This study required computer implemented analysis of sequences often containing thousands of images. However, analysis of one sequence never required more than a few minutes using a desktop computer (Apple iMac27with a 3.5 GHz Intel Core i7 CPU).

Observation and analysis of moving blood cells are facilitated by the fact that the cellular body and enclosing tunic of the young tunicate are transparent and have refractive indexes close to water, so images are not distorted. The stain Neutral Red was used to increase contrast of cells, and it may have only slight specificity for blood cells.

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The pixels of moving cells are selectively observed if sequential video frames are subtracted since the images of stationary cells and other structures cancel in the subtraction. In such a difference image positive (black) pixels are seen where a cell has moved into new locations, and negative (white) pixels are seem where cells have moved from old locations. The use of difference images to follow movement is almost the definition of movement, a change from one image to another, and is basis of many algorithms (Vennemann et al. 2007). Determination of the total number of black pixels in each frame is sufficient for some uses, but it is usually desirable to group contiguous pixels into clumps, which represent images of moving cells.

The low density of cells in the blood of tunicates makes it possible to follow individual cells even in large vessels and relatively high cell numbers because the negative and positive image pairs of individual cells do not usually overlap the images of other cells. However, identification of two sequential cell images as a path of a single cell depends only on the proximity of the sequential images and the absence of other nearby images. In any dense collection of moving cells there are likely to be some errors in assignments of old and new cell images.

It is often useful to collect data selectively from specified regions of the image, which can be done using a mask. A paint program, e.g. Adobe Illustrator (Adobe Systems, Inc.), is used to paint a white segment over the desired area on a black background and this image is then converted into a logical array in which white is true. In the actual scanning program, the acquisition of data from each pixel is then tested against this array. This allows the greatest flexibility in the shape of masks, while minimizing computational overhead.

Results

Metamorphosis and growth

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Within 2-4 hours after the tadpole has attached to a solid surface, the long tail has been adsorbed to produce a more compact animal. In the next 1-2 days the solid body expands to form an internal hollow branchial basket surrounded by a tubular body wall. During this time the internal parts of the animal rotate almost 180 degrees so the anterior portion, the oral siphon (the opening to the branchial basket), is pointing away from the attachment point. Once the branchial basket is pumping water through the mucus net, filter feeding begins and the animal progressively grows in size. As seen in Fig. 1 the exterior lengths of the tunicates used in this study increase from about 0.3 mm to 4 mm in 37 days.

2.5 days: heart activity but no circulation

A tunicate 2.5 days after attachment is seen in Fig. 2. The heart moves in an irregular manner. Blood cells are visible to the eye, but their motion is limited and erratic, and could not be reliably followed by the image-pair software used in this study. There is no real circulation, and no documentation of the limited blood cell movement is presented here.

6.5 days: circulation established

A tunicate 6.5 days after attachment is seen in Fig. 3, which is frame number 25 of a video. The previously dense material adsorbed from the tail is now incorporated into visceral organs, and a vigorous blood circulation is created by the rhythmically contracting heart. In the adult animal the heart is a long tube and an obvious peristaltic constriction pumps the blood, but at this early stage, the heart appears as a compact contracting ball, obscuring its structure.

The red arrow in Fig. 3 points to a dark blood cell in the endostyle region which we will subsequently find is moving. Fig. 4 is a difference image: frame 25 minus frame 26. Pixel intensities in 8-bit grey-scale images have a range of 0 to +255, and thus a difference image has a range of -255 to +255. To display this range in Fig. 4 white represents -255, grey 0, and black

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+255. The white-black doublet at the tip of the red arrow represents one cell in frame 25 which moves about one cell diameter to the right in frame 26. The white (negative) blob is the cell in frame 25 and the black (positive) blob is the cell in frame 26. In this figure the power of the subtraction algorithm in revealing moving cells is dramatic, but all the stationary cells are still present even if invisible, and if they overlap moving cells they will modify the difference image by obscuring the cells in one or both of the frames. Thus as blood cells flow through the tunicate they can be temporarily hidden by individual stationary cells and clumps of cells in organs.

An enlargement of the region around the difference image of the moving cell is seen in Fig. 5. These blood cell images have a maximum diameter of about 5 pixels which represents a diameter of about 9 u at the magnification used in this video (1.7 u / px). There are several types of blood cells in *Ciona* with diameters reported to range from 3.5 to 6.5 u (Millar 1953), giving an average diameter of about 5 u. The apparent diameter of the cells in these difference images is larger than their actual diameter since the cells are represented by a few pixels and any defect in the optical system or smearing by motion will increase the apparent diameter. The distance between the centers of the old and new cell image is approximately 5.5 pixels, or 9.4 u. Since frames are obtained 30 times a second, this represents a speed of 280 u / sec. These cell images may appear crude due to the small number of pixels they contain, but the x and y values of cell centers can be computed to subpixel resolution using an intensity weighted sum over the image, which increases the resolution by about the square root of the number of pixels, or 3-5 fold for cells of 9-25 pixels.

Pulsation of circulation easily measured

The flow of blood in *C. savignyi* is highly pulsatile, as it is in the ascidian *C. inflata* (Konrad 2016). The frame 25-26 difference image described in the previous section was not selected at

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random, but rather because blood was moving rapidly in the two source images to produce a dramatic example of a difference image. The pulsatile nature of blood flow throughout the entire animal can be easily seen by merely plotting the total number of black pixels in difference images against frame number (Fig. 6). The number of black pixels changes about 5 fold every 25 frames, or 0.83 sec. This represents the heartbeat, and demonstrates that blood cell motion is in approximate synchrony throughout the tunicate. At the peaks the image pairs of rapidly moving cells are separated the most and thus the old and new cell images cancel each other the least and the number of black pixels is the highest, while at the troughs cancelation between cell pair images is the highest and there are fewer black pixels.

Following individual blood cells

Much more information can be obtained by resolving and following individual cell images in each difference frame. This is accomplished by first collecting chords of contiguous hit points (pixels with intensity greater than a threshold) along lines of constant y, and then collecting vertically overlapping chords to define cells. In Fig. 7 moving cells from 984 difference frames are displayed as uniform red circular icons superimposed on a grey-scale image of the animal.

An image of the tunicate in which moving cells are represented as circular icons reveals circulation paths, but gives no indication of speed and direction, i.e. velocity, of the flow. However, if image pairs are represented as tadpole icons, with a circular head at the position of the cell in the second frame, and a tail extending to its position in the first frame, they can be imagined as swimming in the direction of cell movement at a speed proportional to tail length. To effectively use tadpole icons it is necessary to limit their density to prevent confusing overlap. A diagram of circulation using non-superimposed tadpoles, about half the total number of image

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pairs, is seen in Fig. 8. Global flow in the animal, indicated by the grey arrows, is in the contra-vertebrate (CV) direction.

Topology of the circulation

The circulatory system in the young tunicate can be approximated as two perpendicular loops. The major loop is in the sagittal plane. During the vertebrate (V) phase of pumping, blood exits the anterior end of the heart, continues in the anterior direction under the endostyle, passes through a loop around the oral siphon, flows in the posterior direction down the large dorsal vessel to the visceral region and splits into three short segments which join at the posterior end of the heart. One of the visceral segments passes through the ovary, another flows over the stomach, while the third has no obvious organ specificity.

The transverse loop is more complex and changes more rapidly during development. Blood flows from the large sub-endostyle vessel up both sides of the branchial basket, typically in a network of several vessels, to meet the large dorsal vessel. When the peristaltic heart reverses direction blood flows in the opposite, contra-vertebrae (CV), direction. CV blood flow is represented by tadpole icons in Fig. 8, while a diagram of the tunicate showing major organs and blood flow as a white path is presented in Fig. 9.

Measuring circulation

To obtain useful values for cell density and speed, i.e. flow rate, it is desirable to study segments between bifurcations in the circulation. Two major vessel segments in the branchial basket, outlined in Fig. 8 in red and green, are the posterior dorsal arc (PDA) from the middle of the basket to the beginning of the three visceral branches, and the posterior ventral arc (PVA) from the anterior end of the heart to the middle of the basket. Numerical parameters describing circulation in these zones are presented in Table 1. The lengths of the segments, number of cells

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and their average speeds are similar. A useful parameter is the number of cells moving out of the end of the segments per second, the cell flow rate, which is 0.37 and 0.35 /sec for PDA and PVA respectively. The difference, 6 percent, is approximately equal to the expected random difference due to the small number of cells (about 400). Thus the total flows are indistinguishable, which is to be expected since these segments are parts of the same loop. The PVA loop under the endostyle has a slightly greater width as seen in this lateral view. As the tunicate grows and develops this vessel becomes even proportionally larger and more complex in cross section, so it could be described as a duct rather than a vessel.

It is important to note that the approximately 400 cell images described in Table 1 were obtained from 984 frames of video, an average of less than one cell image for every two frames. This low density facilities matching image pairs from a single cell in sequential video frames, however, it requires long observation times to quantitate circulation, particularly in smaller vessels.

11 days: more vessels form accross the branchial basket

The flow of blood cells in a tunicate 11days after attachment is seen in Fig. 10. The animal is rotated slightly so the dorsal vessel is closer to the observer and thus more visible while the ventral vessel and endostyle are hidden. Only the side closest to the observer is in focus. There are three rows of stigmata along the axis of the animal, and at least one row has more than one stigmata. Blood flows out from one location on the dorsal vessel and then fans out into 3 or 4 transverse paths across the stigmata before joining the endostyle again at one location.

Path of one cell

In Fig. 10 the proximal side of the vessel ring around the oral siphon appears as a long straight segment in a very clear part of the tunicate body where the density of moving cells is low and it is thus possible to follow individual blood cells through many video frames. The path of one cell

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is displayed in Fig. 11 as a series of dots, one for each frame, with the dot color indicating speed. A plot of speed versus frame number is displayed in the right corner of the Figure. The ratio of maximum to minimum speeds in the peaks along the path ranges from about 2 to 3.

20 days: even more vessels grow around the branchial basket

Circulation in a 20-day old tunicate is seen in Fig. 12. The animal is rotated in the opposite direction to that of Fig. 11, so the dorsal vessel at the top is out of focus, but the endostyle along the bottom can be seen in considerable detail. Blood flows between the endostyle and dorsal vessel in several parallel vessels, and in several places blood flows in a transverse direction between between stigmata, which are still oriented in the transverse direction. Blow flow along endostyle occurs in either several vessels or in a duct with a complex cross section.

37 days: blood flow around stigmata

By 37 days the tunicate has grown almost ten-fold to a length of slightly more than 10 mm. Fig. 13 is a 2.4 x 1.6 mm field of view at the anterior end of the endostyle. As characteristic of tunicates of this age, stigmata are oriented in a transverse direction and are often longer than 500 u, approximately the total length of the 6.5-day old tunicate seen in Fig. 3. In the previous Figure we saw blood flow in several locations between transverse vessels, but in this animal there is more extensive flow in the longitudinal direction so there is essentially flow around the entire circumference of stigmata. Thus, at this stage the blood flow in the branchial basket is clearly two dimensional.

Blood flow in the branchial baskets of younger tunicates may also be two dimensional, but it is perhaps just not so obvious since it is only feasible to follow a modest number of blood cells with the methods used here.

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Discussion

This report uses difference images, the result of subtraction of sequential video frames, to reveal moving blood cells in transparent tunicates. The simple and inexpensive implementation of this method, which has evolved from and is similar to the work of many others, has been described in detail not only to give perspective to the picture of blood circulation presented in this report, but to encourage application to other studies. It could be useful in the study of heart function, movement of appendages, mapping paths of animals, etc.

In the present study the method is facilitated by the low density of blood cells, so that in the 1/30th of a second between successive video frames, a typical tunicate blood cell moves a short distance relative to the average distance between neighboring blood cells. Thus the new position of a cell can be associated with its old position, and not confused with the more distant position of another cell. However, the low density of blood cells typically requires observation times of 10 to 100 seconds (300 to 3000 images) to produce a complete picture of circulation.

Motion of blood cells in *C. savignyi* is very pulsatile, as was also observed in the ascidian *C. inflata* (Konrad 2016). This means that slow blood cells may be "lost" using the frame subtraction algorithm if they move only a small fraction of a cell diameter between video frames. In addition, blood cells can move behind dense cell masses, and may be too close or distant from the focal plane to be detectible. Thus the best images of blood flow throughout an entire animal are obtained in young tunicates, when the animal is transparent and small, so most of the animal is in focus and the path of circulation is simple. Of course circulation can be mapped in larger tunicates for specific regions that are within one focal plane, e.g. Fig. 13, and it should be possible to stitch together the results for several regions to produce a larger map.

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Early blood circulation in the tunicate

Circulation is robust 6.5 days after attachment, and moves through two perpendicular loops. The major loop is in the longitudinal (sagittal) plane. During the vertebrate (V) flow phase blood exits the anterior end of the heart, moves under the endostyle in a large vessel, passes around the oral siphon through a loop, flows down the large dorsal vessel to the visceral region and splits into three short segments which join at the posterior end of the heart. One of the visceral segments passes through the gonad, another flows over the stomach, while the third has no obvious organ specificity.

Multiple secondary loops in the transverse plane allow blood to flow from the ventral to dorsal vessel across both sides of the branchial basket, which occupies most of the area in a lateral view of the tunicate. Blood flows through a two dimensional mesh of channels in the branchial basket that changes in geometry and complexity as the animal grows and develops. Initially the vessels form a fan, with a single connection to the two major vessels. As the tunicate grows and develops the flow becomes a rectilinear net. In older animals it is clear that blood cells move in both transverse and longitudinal directions to create circulation around the edges of individual stigmata. Thus the branchial basket can be considered a flat and wide two-dimensional duct with embedded holes (stigmata).

Blood flow pulsates in phase throughout the animal. The time required for a cell to complete one cycle around the circulation system of the 6.5 day-old tunicate is about 11 seconds, more than 10 fold less than the time between heart reversals. Thus, cell transit times and heart reversals are very separate processes.

The race to feed

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Metamorphosis of the non-feeding tadpole with no blood circulation to an adult tunicate is a race to convert the approximately 2,000 cells in the tadpole to an animal that can filter-feed on plankton before its internal nutritional resources, mainly cells digested by apoptosis of the tail, are exhausted. Much of the process is differentiation and movement of precursor cells, but new cells must also play a part. The newly formed brachial basket must hold and transport the mucus net produced by an endostyle and the embedded cilia lined stigmata must pump water through this net. Blood circulation is needed to transport nutrients from the stomach to the endostyle to continually make the net and to the cilia around the edges of the stigmata to supply energy for moving water through the net. In contrast to vertebrates there is neither a placenta or yolk, and thus there is no circulation associated with these sources of nutrition.

At 6.5 days after attachment blood circulation is seen and the first functional stigmata appear along transverse vessels between the large ventral and dorsal vessels. The transverse vessels initially increase in number by splitting, to form a fan between the ventral and dorsal vessels. As the tunicate grows the number of transverse vessels increases and the geometry becomes more rectilinear, so that the the transverse vessels are parallel. The number of stigmata also increases, they are larger, and are elongated along the longitudinal direction.

Early blood circulation in the fish

The vertebrate that seems closest to the tunicate is the fish. As with tunicates it is marine, evolved earlier than terrestrial animals, and is cold blooded. As with tunicates the heart of the fish is a straight tube which pumps blood directly to gills. Initially, like the tunicate, it pumps by peristaltic contractions(Bakkers 2011), but then develops fixed valves and several chambers, and pumps by contraction. However, blood flow in the fish, as in all vertebrates, is always in one direction.

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The race to swim

The developmental race for the young fish is to produce a large muscular tail, eyes, and a brain in order to escape predators and later to capture prey, and there is nutrition in the yolk for this purpose(Isogai et al. 2001). Thus the major function of early circulation is to transport nutrients from the yolk to the head and tail to allow rapid cell growth and division. In contrast, the adult tunicate has has lost its tail, most of its neurons, and its primitive eye, as all are useless as a sessile animal. Early circulation in the fish is a loop from heart to the gill arch in the head, and then down the dorsal edge of the tail, returning along the ventral edge back to the heart. Buds along the parallel dorsal and ventral vessels in the tail grow toward each other between the myotomes, join and form a network of parallel vessels. During this stage of development there is but one gill arch. The fish must eventually develop larger gills to provide sufficient oxygen for the muscular activity of an adult animal, but the gills will never have the proportional size or play as direct a role in food capture as they do in the tunicate.

Analogous vascular buds might form on the dorsal and ventral vessels of the tunicate branchial basket, but they would not be visible with the methods used in this report until they merged and actual blood flow was established.

In both tunicates and fish blood flow is highly pulsatory. In this report a young, sessile tunicate 6.5 days post attachment and 0.4 mm long, was shown to have a peak speed of blood flow in the major vessels of about 0.4 mm/s. In a fish 3-5 days post fertilization, approximately 2 mm long, peak blood speeds were found to be about 1 mm/s(Watkins et al. 2012). Thus blood speeds are comparable in young tunicates and fish.

Comparison of the branchial basket of the tunicate and the gill of fish

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The branchial basket of the tunicate would seem to be analogous to the gills of the fish because the gross anatomy is similar and in both organs water flows in through the oral siphon (mouth) passes through the stigmata of the branchial basket (gill arches) and exits via the atrial siphon (gill slits).

However, in the tunicate the endostyle and the stigmata lined with cilia are defining functional components of the branchial basket and a major function of blood flow must be to supply nutrients to these organs. The branchial basket is made of two layers of cells, with spacing defined along the periphery of the stigmata by seven rows of ciliated cells, at least 70 microns(Burighel & Cloney 1997; Martinucci et al. 1992). Blood flows through this two dimensional duct from the ventral to dorsal vessels, but also longitudinally, so that there is flow around the periphery of stigmata.

In contrast, the major function of the gills of fish is passive, to facilitate diffusion of oxygen from water to blood and carbon dioxide from blood to water. The functional unit of the gill is the laminae, a lobe of two parallel layers formed by pillar cells through which blood moves(Olson 2002). A portion of each pillar cell, as its name suggests, is on each side of the double layer, and thus this cell determines the separation between layers, which is slightly larger than the diameter of a blood cell, or about 15 microns. Thin laminae facilitate diffusion of gases between blood and water.

Thus, the branchial basket of the invertebrate tunicate and the gills of the vertebrate fish have different functions, have a different microanatomy, and a different histology. These differences complicate construction of a path for the evolution of the branchial basket and gills from a common ancestor.

Conclusions

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Attachment of the tunicate larva tadpole to a solid surface starts metamorphosis to the adult body plan. Blood circulation begins after 3 to 6 days with a sagittal loop from the anterior end of the heart, up the ventral edge of the endostyle to a loop around the oral siphon, and down the dorsal edge of the branchial basket to split into three short branches in the visceral cavity which rejoin at the posterior end of the heart. Blood also flows from the ventral to dorsal vessels along the sides of the branchial basket. As the tunicate develops the branchial basket grows and the number of paths of blood flow across the basket increases to create a rectilinear mesh, with flow around all sides of the stigmata. Blood flow pulses in phase throughout the animal, synchronous with heart action, consistent with a closed hydraulic system. The velocity of blood flow is comparable with values seen in the developing fish.

References

Augustin H, and Koh GY. 2017. Oganotypic vasculature: from descriptive heterogeneity to functional pathophysiology. *Science* 357:771-782. dx.doi. org/10.1126/ science.aal2379

Azumi K, Sabau SV, Fujie M, Usami T, Koyanagi R, Kawashima T, Fujiwara S, Ogasawara M, Satake M, Nonaka M, Wang HG, Satou Y, and Satoh N. 2007. Gene expression profile during the life cycle of the urochordate Ciona intestinalis. *Dev Biol* 308:572-582. 10.1016/j.ydbio.2007.05.022

Bakkers J. 2011. Zebrafish as a model to study cardiac development and human cardiac disease. *Cardiovascular Research* doi: 10.1093/cvr/cvr098.

Berna L, and Alvarez-Valin F. 2014. Evolutionary genetics of fast evolving tunicates. *Benome Biol Evol* 6:1724-1738.

Berna L, Alvarez-Valin F, and D'Onofrio G. 2009. How fast is the sessile ciona? *Comp Funct Genomics* 2009:e875901:875-901. 10.1155/2009/875901

Broeke J, Perez JMM, and Pascau J. 2015. *Image Processing with ImageJ, 2nd ed*. Birmingham-Mumbai: Packt Publishing.

Burighel P, and Cloney RA. 1997. Urochordata: Ascidiacea. In: Harrison FW, and Ruppert EE, eds. *Hemichordata, Chaetognatha, and the invertebrate chordates*. New York: Wiley-Liss, 221-347.

Chiba S, Sasaki A, Nakayama A, Takamura K, and Satoh N. 2004. Development of Ciona intestinalis Juveniles (Through 2nd Ascidian Stage). *Zoological Science* 21:285-298.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26556v1 | CC BY 4.0 Open Access | rec: 21 Feb 2018, publ: 22 Feb 2018

Christiaen L, Wagner E, Shi W, and Levine M. 2009. The Sea Squirt Ciona intestinalis. *Cold Spring Harbor Protocols*.

Cirino P, and Brown E. 2014. Protocol for fertilization tests in Ciona intesinalis. *Available at* www.assemblemarine.org / *assets* / *Assemble-JRA1-Protocol-02.00.pdf*.

Davidson B. 2007. Ciona intestinalis as a model for cardiac development. *Semin Cell Dev Biol* 18:16-26.

Davidson B, Shi W, Beh J, Christiaen L, and Levine M. 2006. FGF signaling delineates the cardiac progenitor field in the simple chordate, *Ciona intestinalis*. *Genes and Development* 20:2728-2738.

Delsuc F, Brinkmann H, Chourrout D, and Philippe H. 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* 439:965-968.

Foundation TE. 2017. Eclipse *Available at* https://eclipse.org/.

Isogai S, Horiguychi M, and Weinstein BM. 2001. The Vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev Biol* 230:278-301.

Karaiskou A, Swalla BJ, Sasakurs Y, and Chambon J-P. 2015. Metamorphosis in solitary ascidians. *Genesis* 53:34-47.

Konrad MW. 2016. Blood circulation in the ascidian tunicate *Corella inflata* (Corellidae). *PeerJ*. https://doi.org/10.7717/peerj.2771

Kriebel ME. 1968. Studies on cardiovascular physiology of tunicates. *Biol Bull* 134:434-455.

Martinucci GB, Dallai R, Burighel P, and Casagrande L. 1992. Ciliary specializations in branchial stigmatal cells of protochordates. *Tissue and Cell* 24:229-241.

Millar RH. 1953. *Ciona*: The University Press of Liverpool.

Monniot C, Monniot F, and Laboute P. 1991. *Coral Reef Ascidians of New Caledonia*. Paris: Orstom.

NIH. 2017. ImageJ, image processing and analysis in Java. *Available at* http://imagej.nih.gov/.

Olson KR. 2002. Vascular anatomy of the fish gill. *J Exp Zool* 293:214-231.

Passamaneck YJ, and Di Gregorio A. 2005. Ciona intestinalis: chordate development made simple. *Dev Dyn* 233:1-19. 10.1002/dvdy.20300

Satoh N. 1994. *Developmental Biology of Ascidians*: Cambridge University Press.

Satoh N. 2016. *Chordate origins and evolution*. *The molecular evolutionary road to vertebrates*. Amsterdam: Academic Press.

Schwerte T, and Pelster B. 2000. Digital motion analysis as a tool for analysing the shape and performance of the circulatory system in transparent animals. *J Exp Biol* 203:1659-1669.

Schwerte T, Uberbacher D, and Pelster B. 2003. Non-invasive imaging of blood cell concentration and blood distribution in zebrafish *Danio rerio* incubated in hypoxic conditions *in vivo*. *J Exp Biol* 206:1299-1307.

Stolfi A, Gainous TB, Young JJ, Mori A, Levine M, and Christiaen L. 2010. Early chordate origins of the vertebrate second heart field. *Science* 329:565-568.

Vennemann P, Lindken R, and Westerweel J. 2007. In vivo whole-field blood velocity measuremant techniques. *Exp Fluids* 42:495-511.

Vogel L. 2013. *Eclipse IDE*. *Java programming, debugging, unit testing, task management and Git version control with Eclipse*: Vogel/a.

Watkins SC, Maniar S, Mosher M, Roman BL, Tsang M, and St Croix CM. 2012. High Resolution Imaging of Vascular Function in Zebrafish. *PLOS One* 7:e44018.

Willert CE, and Gharib M. 1991. Digital particle image velocimetry. *Expt Fluids* 10:181-193.

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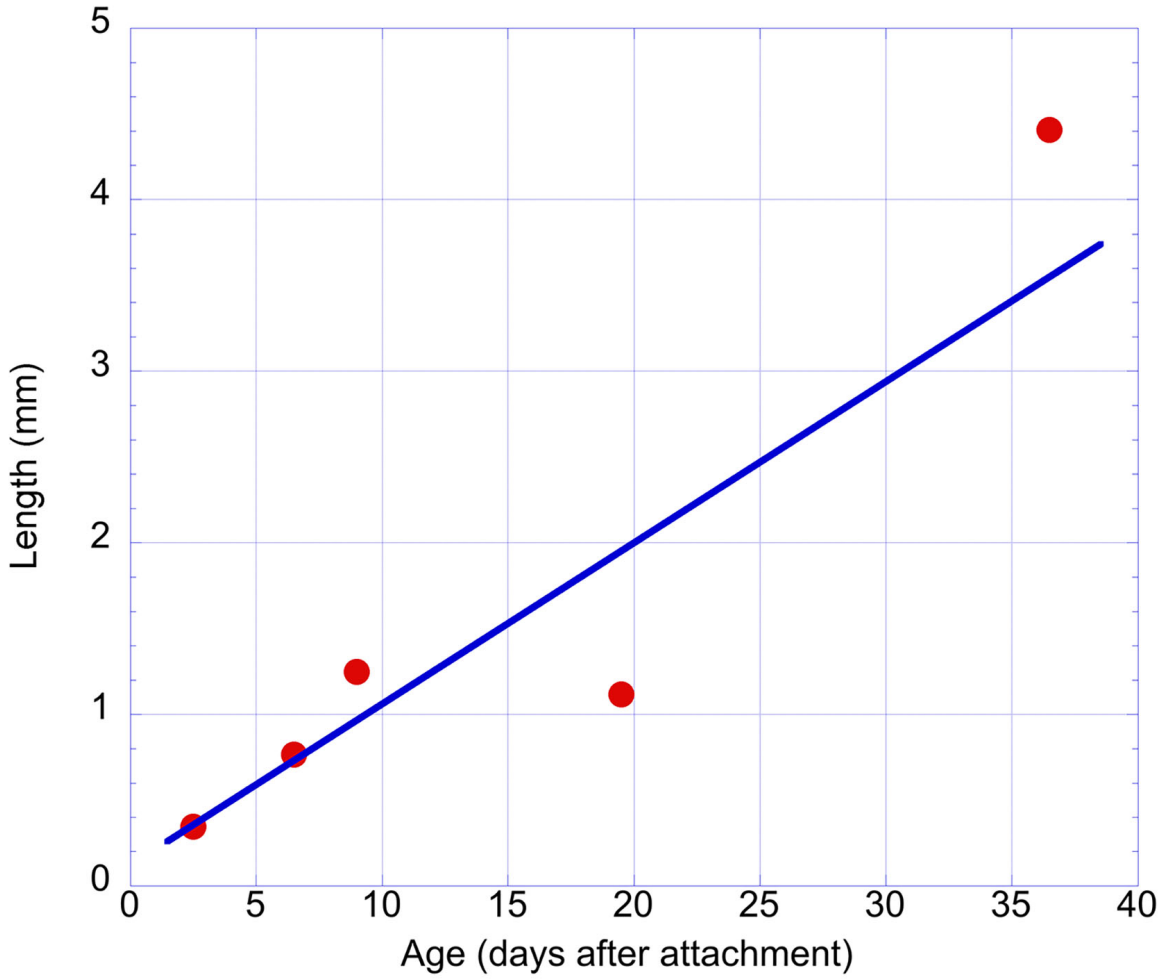

Figure 1. Lengths and ages of tunicates.

Blood circulation was visualized in five tunicates of increasing ages (days after attachment). This graph is an index to the specific tunicates described in this report, not a growth curve for tunicates. The length of an animal is not always proportional to volume, since shapes are variable. As an example, the animal 19.5 days after attachment was almost spherical, while the animal 36.5 days after attachment was very elongated.

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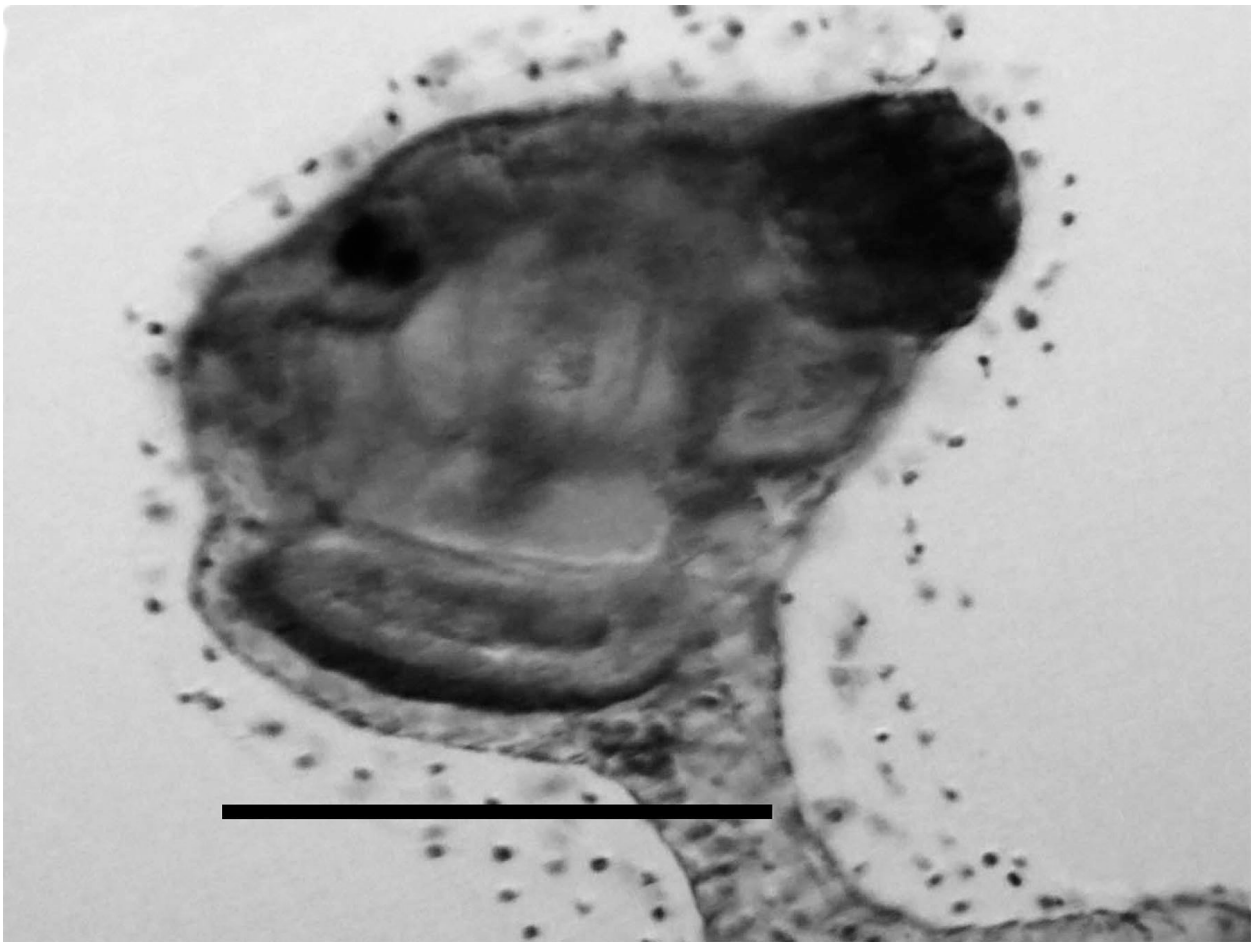
506 

Figure 2. A tunicate 2.5 days after attachment.

507 The solid, long, thin larval tadpole has become two concentric hollow cylinders, closed at the
508 posterior (right) end. The dense remnant of the tail, at the upper right of the image, will become
509 the viscera and gonads of the adult. The tunicate is attached to the substrate by the stalk
510 projecting downward at the lower center of the image. The few blood cells move in short erratic
511 paths. The scale bar represents 0.5 mm.

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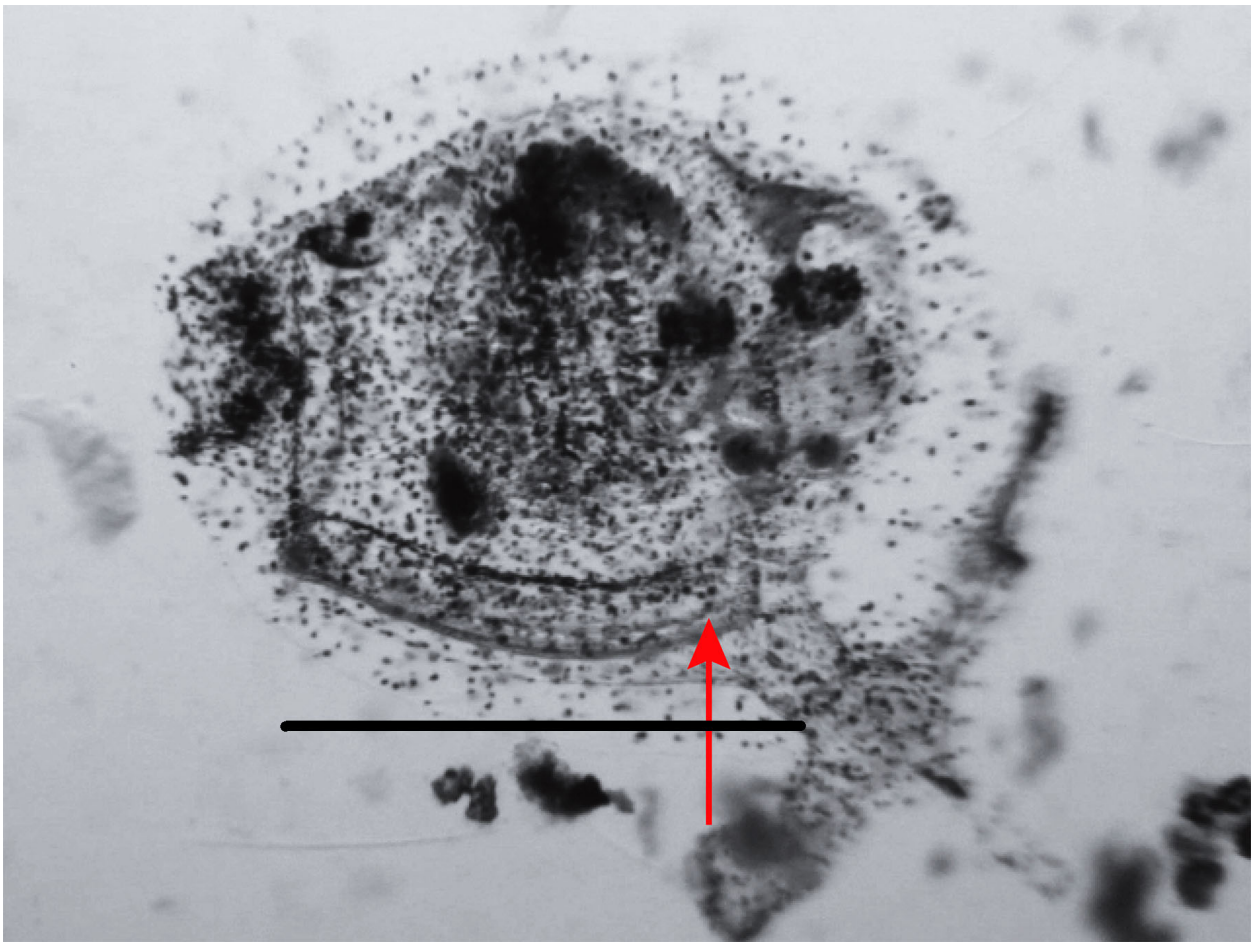
513 

Figure 3. A tunicate 6.5 days after attachment with a moving blood cell marked by the red arrow.

514 The great majority of cells in this image are stationary, but about 10 will be seen to be moving.

515 One rapidly moving large cell is indicated by the red arrow. The scale bar represents 0.5 mm.

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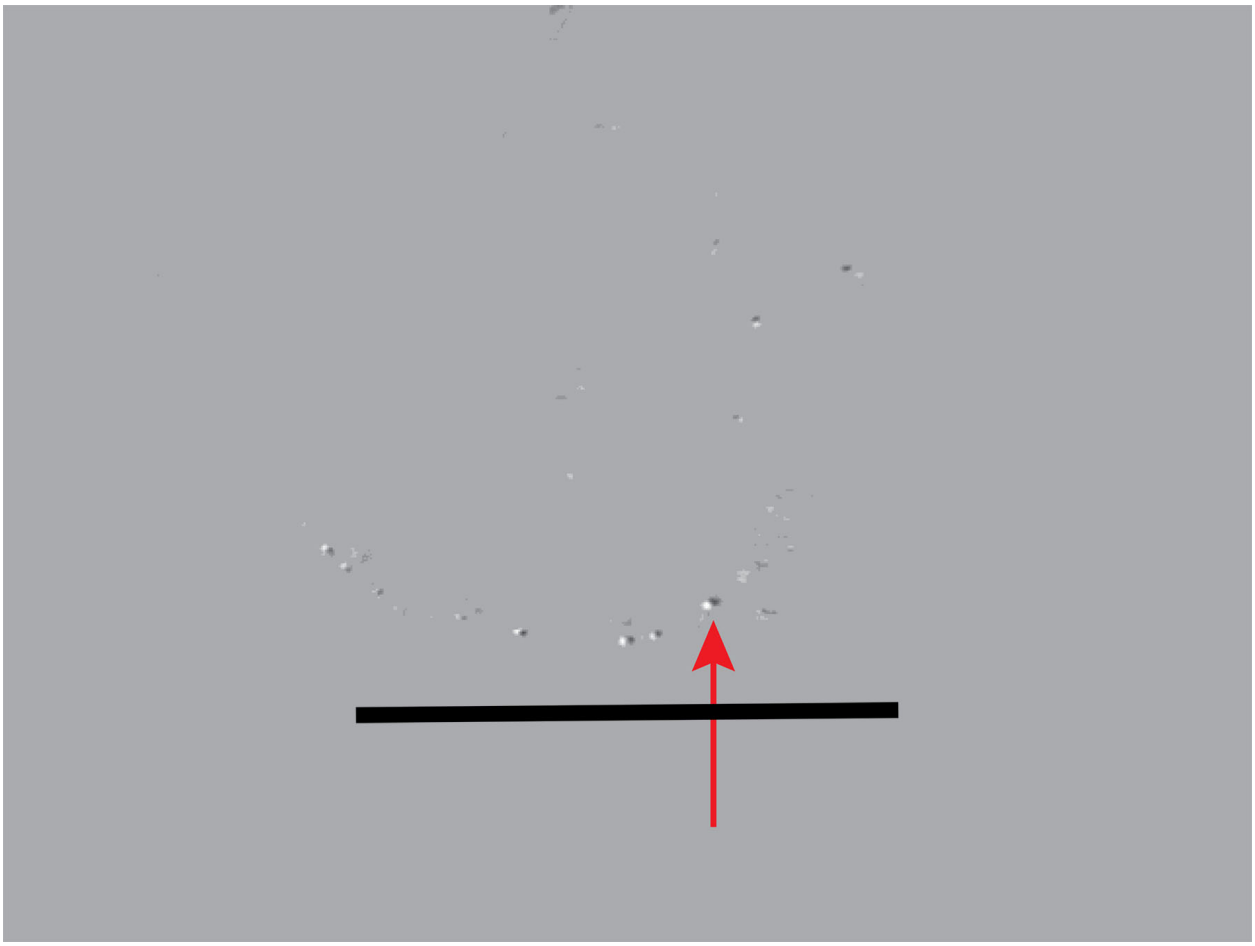

Figure 4. The difference image reveals moving cells.

The image seen in the previous figure was subtracted from the subsequent image in the video sequence to produce this difference image. Intensities in a difference image have a range of -255 to +255, and in this image negative intensities are black, zero intensities are grey, and positive intensities are white. The moving cell at the tip of the red arrow is represented by a black spot in the old position and a white spot in the new. The scale bar at the bottom represents 0.5 mm.

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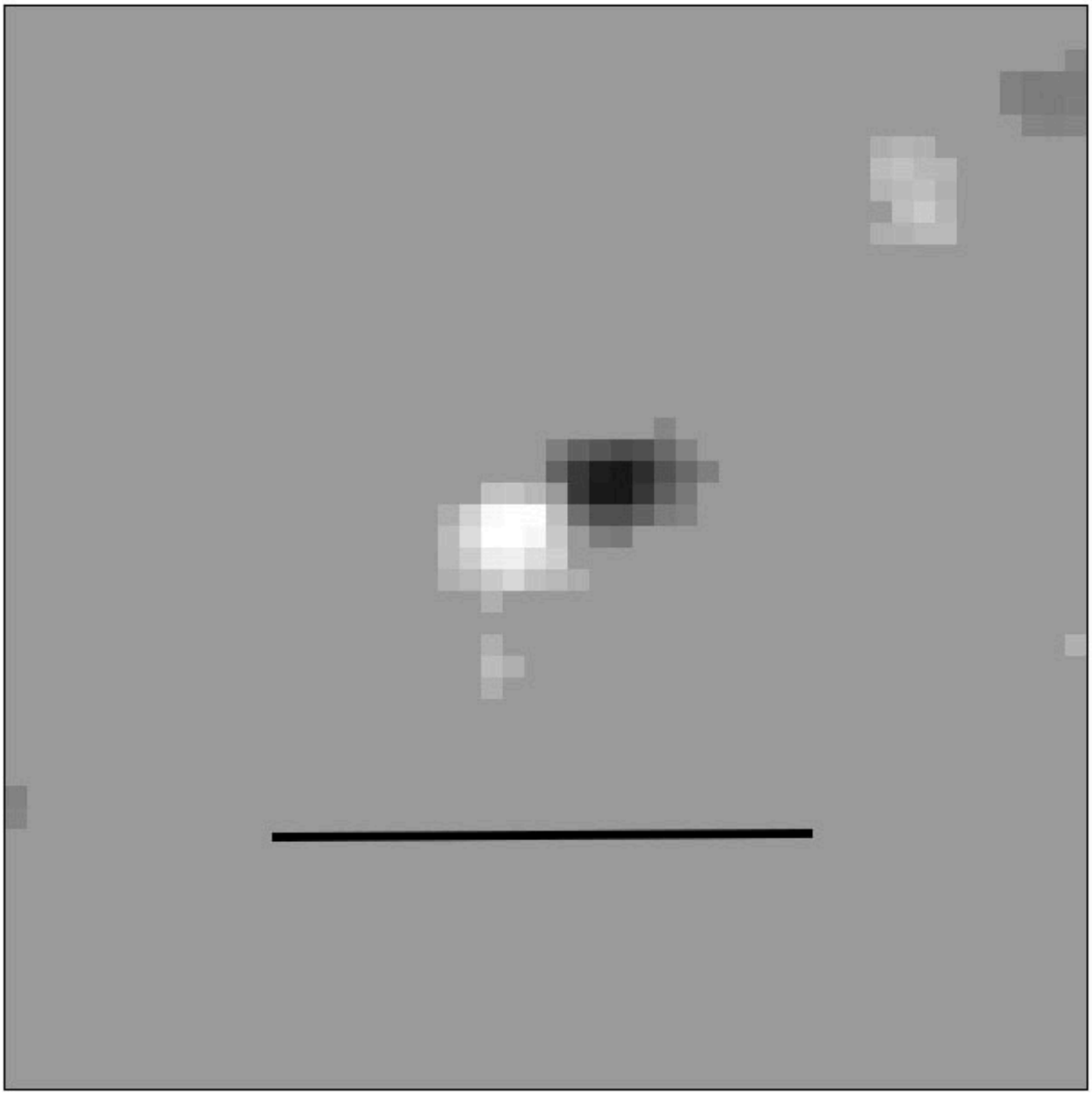

Figure 5. Difference image of a image pair at pixel resolution.

This is an enlarged view of the cell image pair at the end of the red arrow in the previous figure. Each pixel in this image represents 1.7 microns in real space. The scale bar represents 25 microns.

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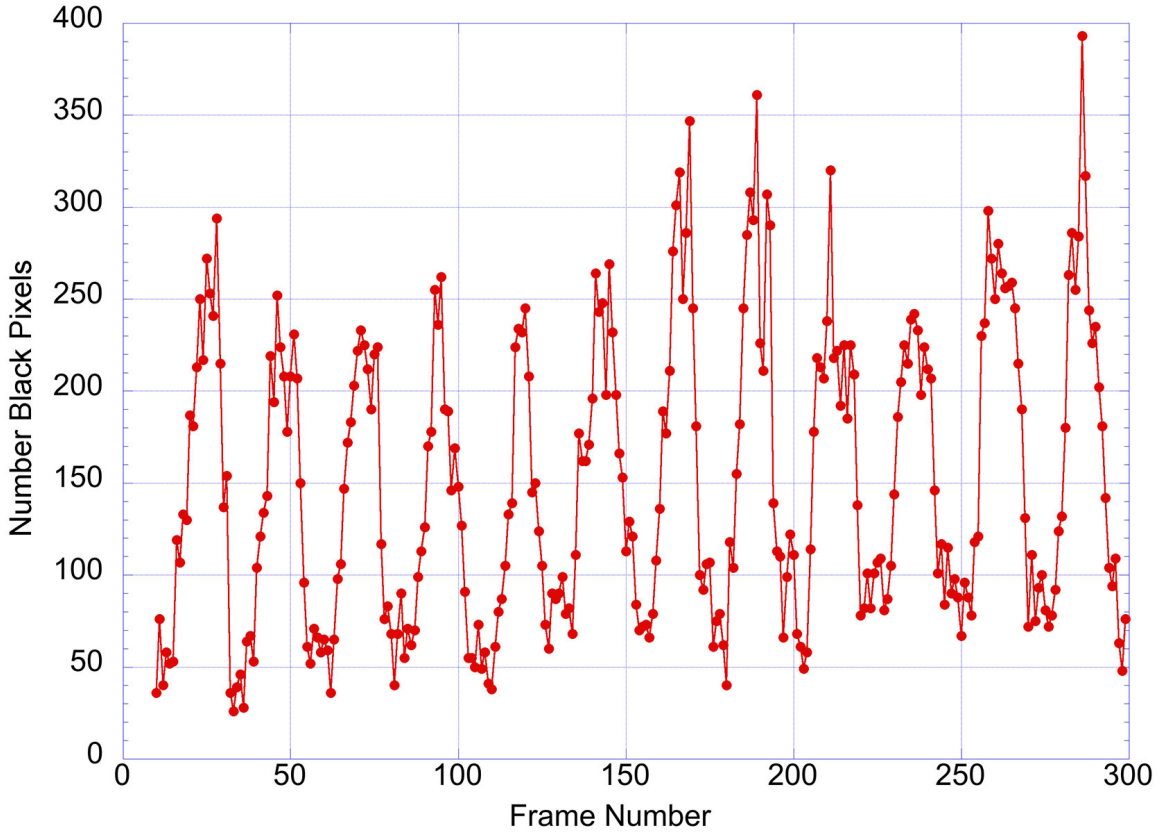

532

Figure 6. Black pixels in sequential difference images.

533 Moving blood cells generate black pixels in difference images. Sequential video frames
534 containing rapidly moving cells generate difference images with many black pixels, while
535 sequential frames with slowly moving cells produce few. Thus the oscillations in black pixel
536 numbers reflect the pulsations in blood speed.

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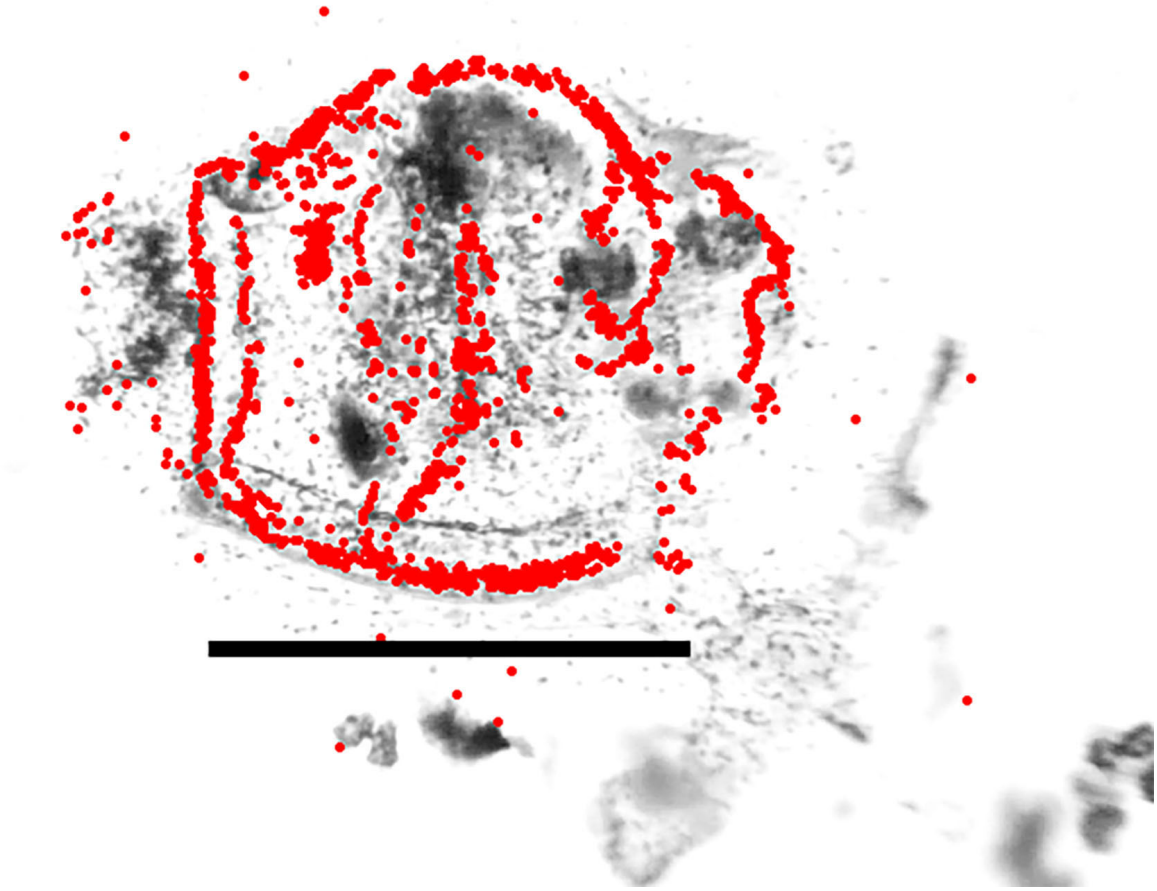

Figure 7. Cumulative difference image maps circulation.

Contiguous dark pixels in individual difference images were grouped into clumps which represent moving blood cells. Each clump was represented by a standard circular red cell icon, and this cumulative image was generated containing all the cell icons from the sequence. The oral siphon (anterior end of animal) is at left, visceral cavity (posterior end of animal) is on the right side, ventral and dorsal sides are at bottom and top respectively. Black scale bar represents 0.5 mm in real space.

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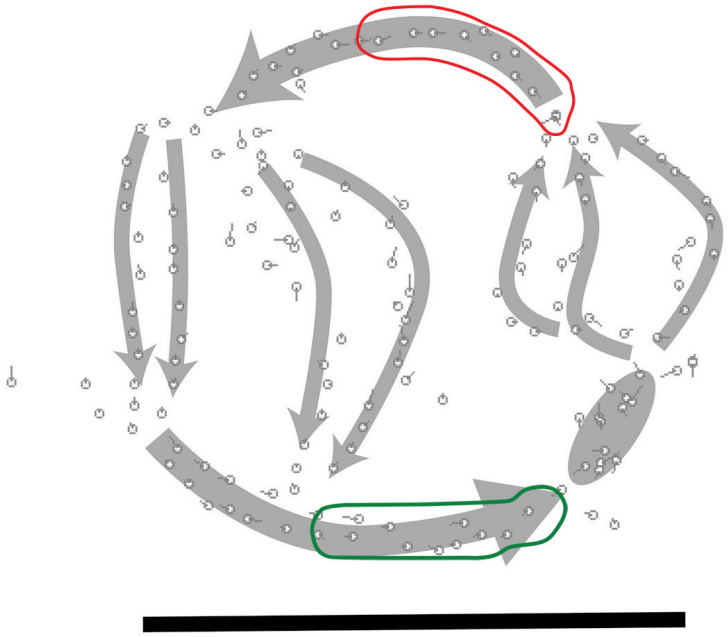

548

549 **Figure 8. Paths and velocities of blood flow.**

550 Moving cells seen in Fig. 7 as circles are represented in this figure as tadpole icons, but cells that
551 overlap have been omitted for clarity. The open head of the tadpole is at the position of the cell
552 in the second frame, and a tail extends to the position of the same cell in the first frame. Thus the
553 length and direction of the tail represents the velocity of the cell. Red and green lines define
554 regions for which cell numbers and speeds are pooled and analyzed in Table 1. The scale bar
555 represents 0.5 mm in real space.

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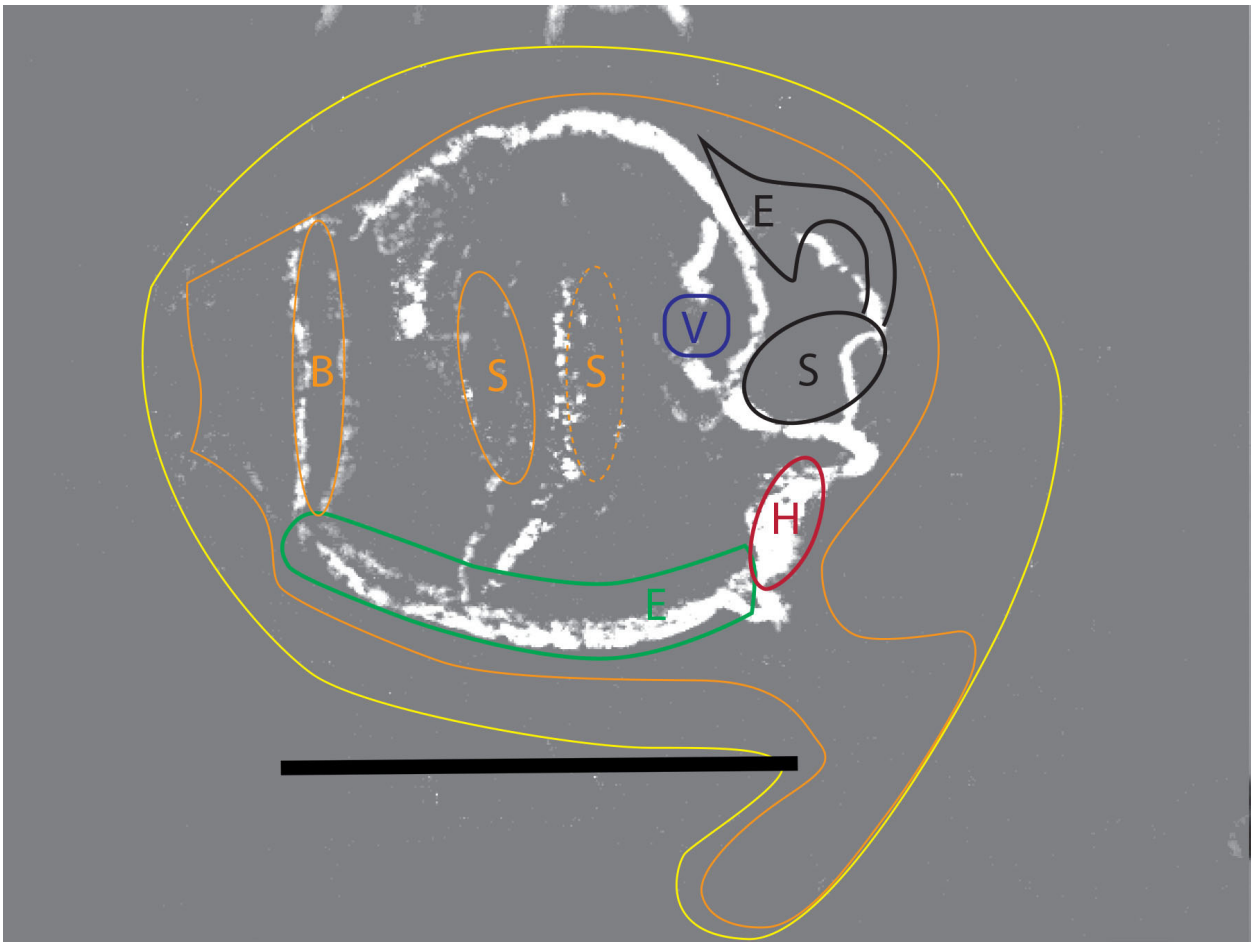

Figure 9. Blood circulation relative to tunicate anatomy.

The profile of the outer tunic is in yellow; the inner body, buccal vessel loop (B) and stigmata (S) orange; endostyle (E) green; heart (H) red; ovary (V) blue; esophagus (E) and stomach (S) black. The path of moving blood cells is in white. Scale bar is 0.5 mm in real space.

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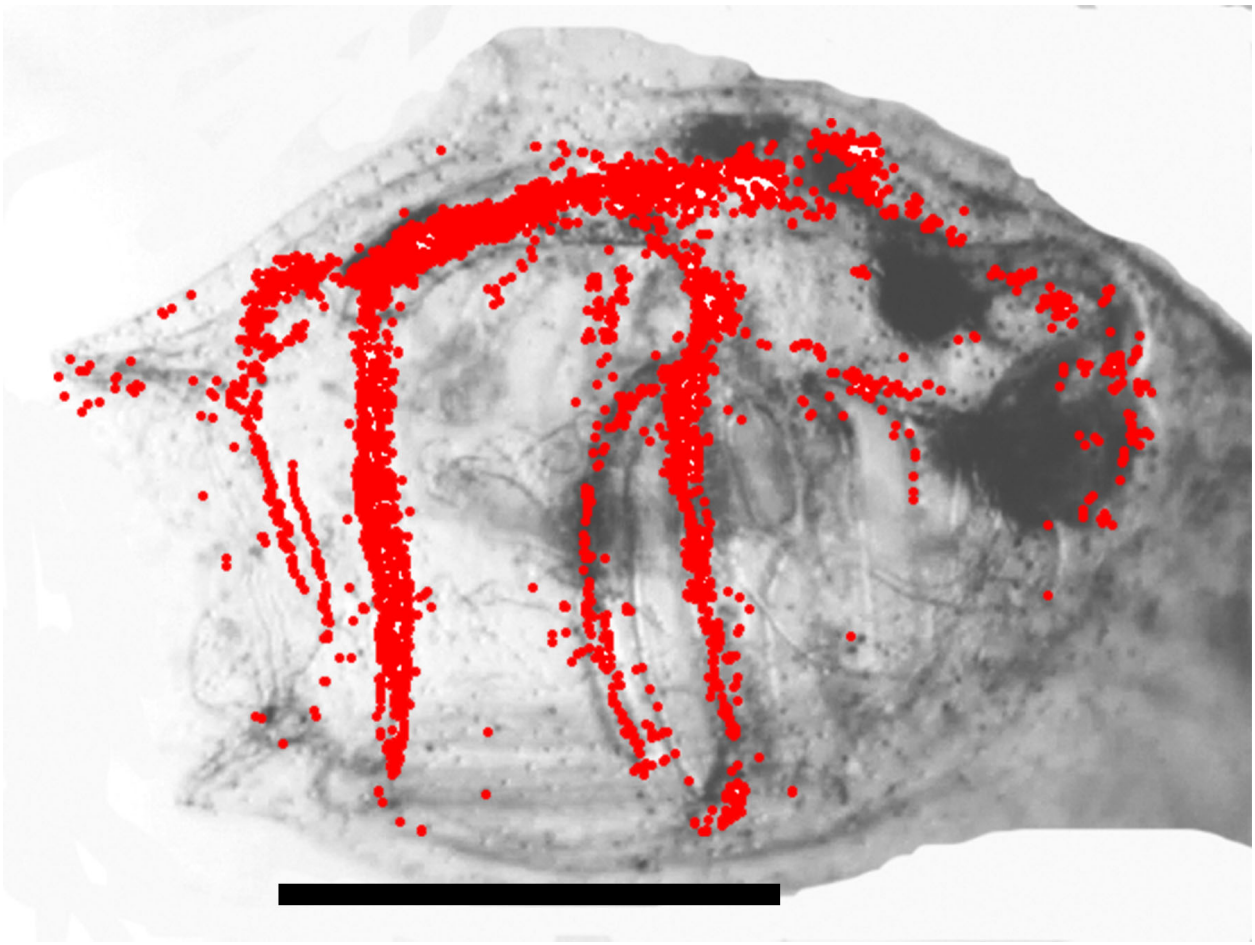

Figure 10. Blood circulation 11 days after attachment.

Moving blood cells are represented by circular red icons. This tunicate is rotated approximately 30 degrees along the body axis, moving the dorsal vessel toward the viewer. Thus the dorsal vessel is prominent, while the endostyle and associated ventral vessel are out of focus and not visible. Circulation from the edges of several stigmata combine before entering the large dorsal vessel. The scale bar represents 0.5 mm in real space.

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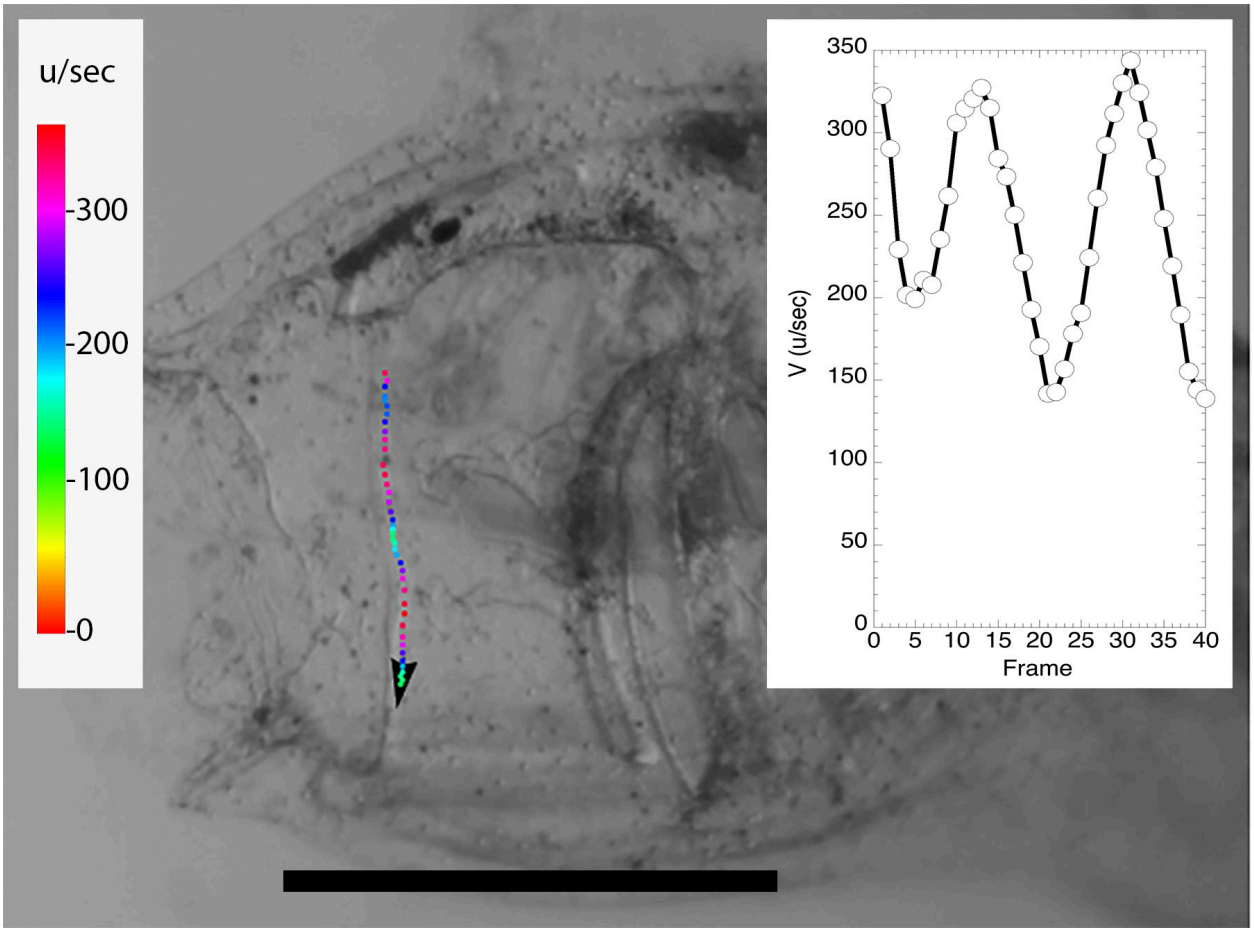

Figure 11. Path and speed of one blood cell.

A single moving blood cell, visible over many frames, was selected from the animal seen in the previous Figure. During 40 frames, about 1.3 seconds, it moved about 0.5 mm down the vessel loop that surrounds the oral siphon, in the contra-vertebrate direction. The speed is pulsatile, changing by a factor of 2 during heart beats. The path is represented by a series of dots superimposed on the image of the tunicate, one per video frame, and thus spacing between dots indicates speed. The dots have been colored to also reveal speed, with a legend in the left panel. A graph of speed versus frame number is displayed in the right panel.

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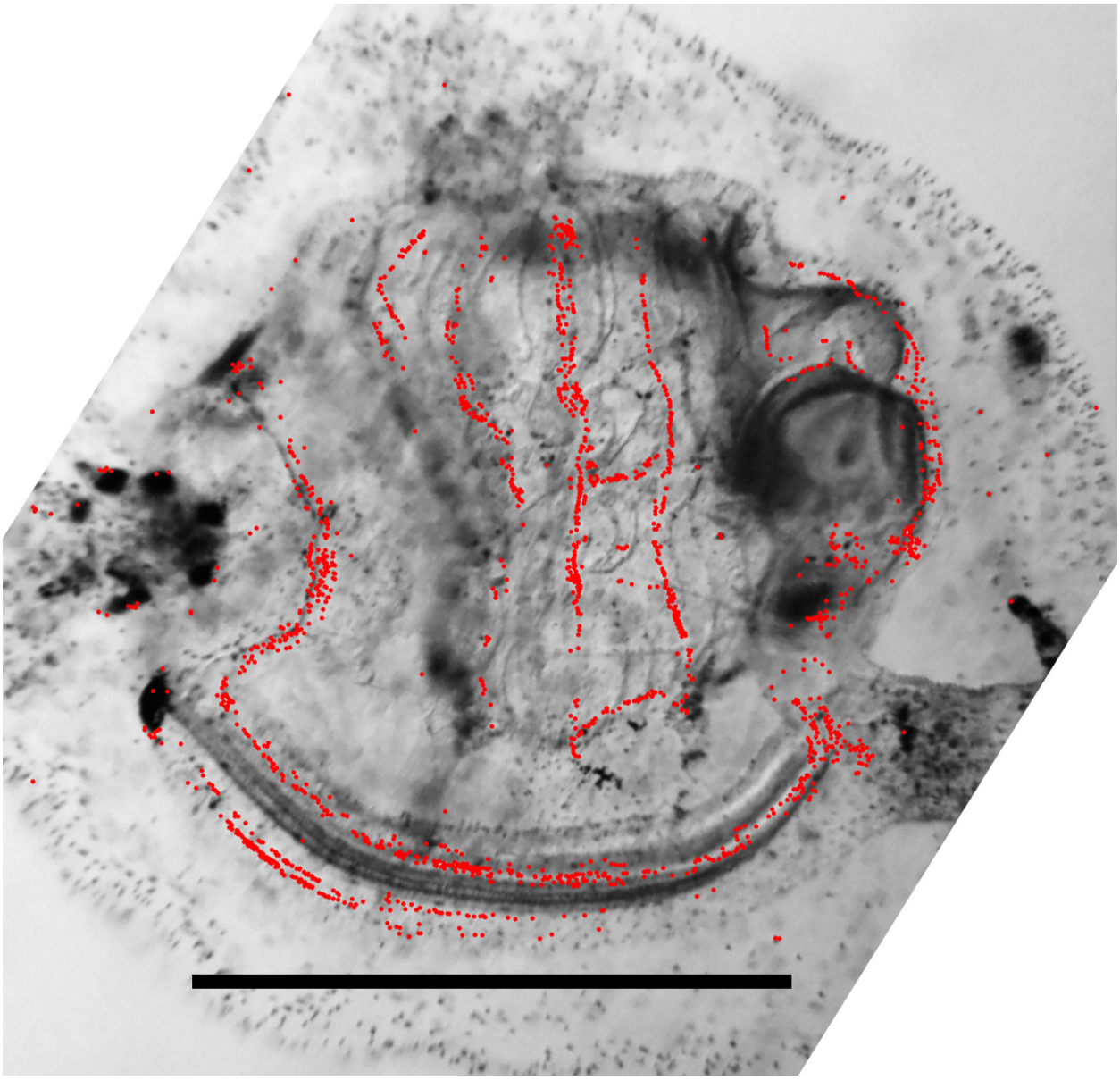

Figure 12. Blood circulation 20 days after attachment.

This tunicate is rotated along the body axis in the opposite direction to Fig. 10 so moving blood cells in the ventral endostyle are in focus, while those in the dorsal vessel are not visible. Blood flow across the branchial basket is more rectilinear than seen in the previous animal. Blood appears to flow along the endostyle in two parallel paths. In other animals of this age the blood channel under the endostyle appears quite large.

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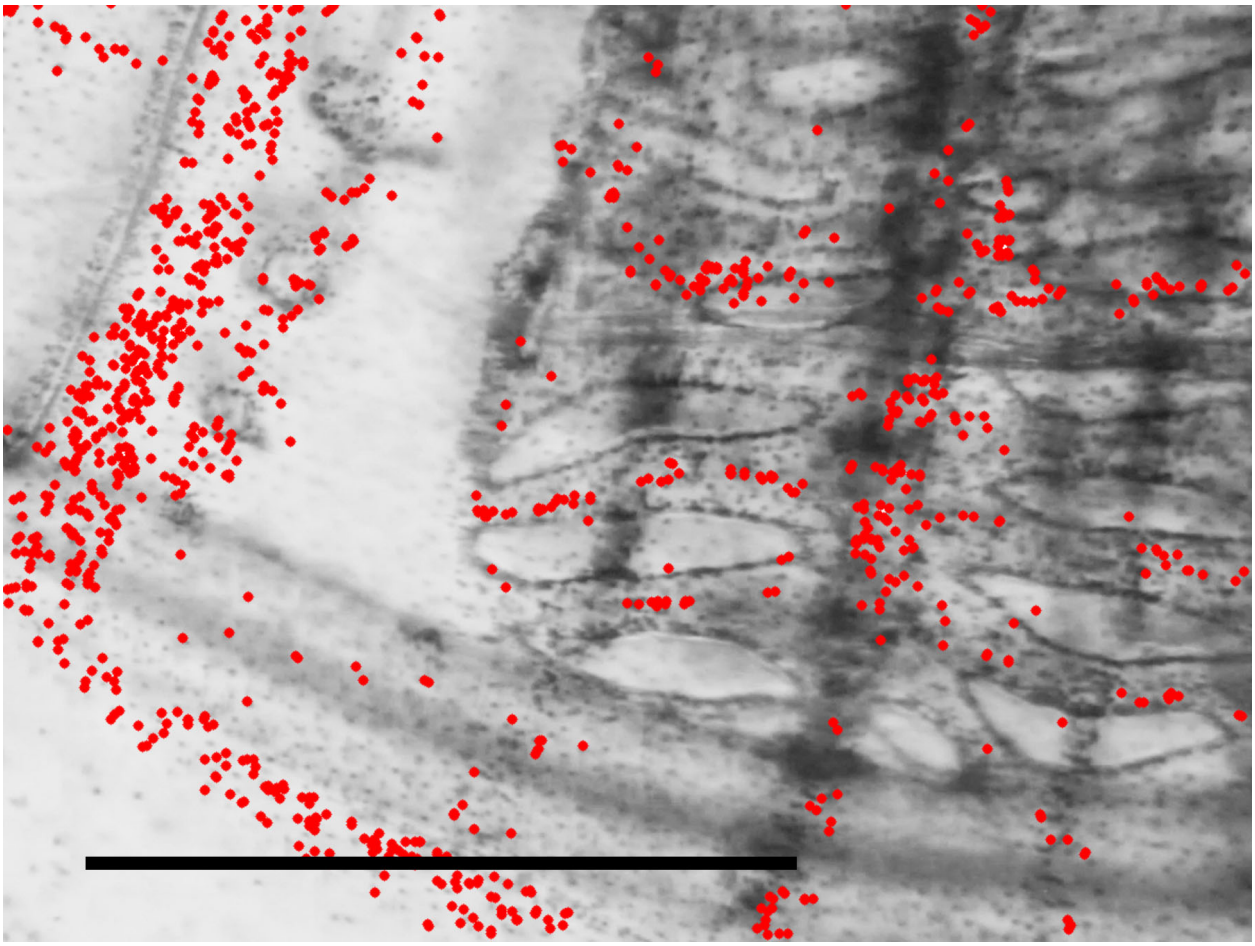

Figure 13. Blood flow around stigmata 37 days after attachment.

The entire tunicate at this stage is 4.4 mm long, but this image is just an enlargement of the ventral-anterior corner of the branchial basket. Blood cells are seen moving vertically along a transverse dark grey bar (inclined at a slight angle from the vertical along the right quarter of the image) which connects the dorsal to the ventral vessel running under the endostyle. Blood cells are also seen moving between the stigmata. The scale bar represents 0.5 mm in real space.

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Parameter	Dorsal (red)	Ventral (green)
segment length (u)	211	238
apparent segment diameter (u)	22	30
total cells followed in 33 sec	489	398
mean cell speed (u/s)	158	206
apparent fluid flow rate (u^3/s)	60,000	146,000
cell flow rate (cells/s)	0.37	0.35

595

596 **Table 1. Blood cell flow through two major vessel segments.**

597 This table summarizes flow during a period of 33 seconds through the dorsal red and ventral
598 green vessel segments defined in Fig. 8. Segment lengths are well defined, but the apparent
599 diameters are only distances between parallel curves bracketing the moving cell images in the 2D
600 image; if the vessel is not circular this value has little meaning. Thus, while the mean cell speed
601 is well defined, the apparent fluid flow rate depends on the vessel being circular. However, the
602 cell flow rate is well defined, since it is just dependent on the number of cells in the segment, the
603 mean speed, and the segment length.

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