The Initiation of Blood Flow and Flow Induced Events in Early Vascular Development

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Abstract

Within a day of gastrulation, the embryonic heart begins to beat and creates blood flow in the developing cardiovascular system. The onset of blood flow completely changes the environment in which the cardiovascular system is forming. Flow provides physiological feedback such that the developing network adapts to cue provided by the flow. Targeted inactivation of genes that alter early blood fluid dynamics induce secondary defects in the heart and vasculature and therefore proper blood flow is known to be essential for vascular development. Though hemodynamics, or blood fluid dynamics, are known to activate signaling pathways in the mature cardiovascular system in pathologies ranging from artherosclerosis to angiogenesis, the role in development has not been as intensively studied. The question arises how blood vessels in the embryos, which initially lack cells types such as smooth muscle cells, differ in their response to mechanical signals from blood flow as compared to the more mature cardiovascular system. Many genes known to be regulated by hemodynamics in the adult are important for developmental angiogenesis. Therefore the onset of blood flow is of primary importance to vascular development. This review will focus on how blood flow initiates and the effects of the mechanical signals created by blood flow on cardiovascular development.

1. Introduction

During embryonic development, blood flow is essential not only to nourish growing tissues but it also provides physiological signals that are required for the development of the blood vessels themselves. The heart begins to beat early on embryonic day 8.0 or E8.0 in mouse embryos [1, 2], and erythrocytes begin to circulate a few hours later at E8.5, equivalent to the 5 somite stage [3-5]. The developing cardiovascular system is exposed to a whole new set of cues when circulation is established. Vascular remodelling is an adaptation of the vascular network to such cues. Physiological feedback occurs through chemical signals, such as hypoxia and nutritional requirements, but also through mechanical stimuli that are derived from blood flow, such as shear stress and circumferential stretch. As soon as the vasculature forms, the network is responsive to the physiological needs of the tissue and capable of remodelling to meet these needs. For this reason, physiological signals are of fundamental importance to vascular development and shape the growing network of vessels as it forms.

Flowing blood creates two forces; shear stress, which is a force that is parallel to the endothelial surface and is related to a fluid's velocity and viscosity, and circumferential strain, which is a force perpendicular to the vessel and is related to the intraluminal pressure. Though the role of both of these forces has been studied extensively in the mature cardiovascular system, much less is known about how these forces affect development in the embryo. We will review here how blood flow initiates and what is known about the role of the early blood flow during development of the cardiovascular system.

2. The Initiation of Blood Flow

In order for flow-related stimuli to even be present, a functional loop for blood flow must first be established. The initiation of blood flow requires several events to come together including the formation of the initial vascular plexus, lumenization of vessels, initiation of heart contraction and the entry of erythrocytes into circulation. These events are tightly regulated and occur within less than a day in mouse embryos (summarized in Table I).

Vasculogenesis is the *de novo* formation of blood vessels. During development, the emergence of the first VEGFR2+ cells occurs at E7.0 [6]. These VEGFR2+ cells are precursors of both hematopoietic and endothelial cells. These early cells migrate from the primitive streak to the proximal yolk sac, forming a structure known as the blood islands. The blood islands are composed of erythroblasts, precursors to red blood cells, and angioblasts, precursors to endothelial cells. Erythroblasts remain confined to the blood islands until much later in development, but angioblasts migrate from blood islands towards the embryo between 0 and 4 somites [6]. As the angioblast population expands, these cells form interconnected cords resulting in a vascular network called the capillary plexus. A cord refers to string of angioblasts lacking a vessel lumen. A second site of angioblast differentiation is present in the embryo proper. These cells aggregate along the embryonic midline to form the dorsal aortae [6]. The location and size of major arteries and veins, such as the dorsal aorta, are reproducible from one embryo to another implying a genetic roadmap. Furthermore, *in vitro* models such as embryoid bodies can be induced to differentiate into a network of vessels reminiscent of the capillary plexus [7]. For these reasons, vasculogenesis is believed to be a developmental program in which physiological feedback does not play as significant a role.

Though a complete network of angioblasts is present by 4 somites, this does not represent a network in which blood can circulate until these angioblastic cords have lumenized. The fusion of intracellular vacuoles within endothelial cells has long been believed to be the mechanism by which the vascular lumens form based on observations in chick embryos by Florence Sabin [8]. In a beautiful series of time-lapse images, the presence and fusion of such vacuoles was followed *in vivo* using the intersegmental vessels of transgenic zebrafish that expressed an eGFP-cdc42wt fusion protein [9]. Though vacuole-like structures are clearly observed in these movies, more recent work has called into question whether these structures truly represent intracellular vacuoles. Blum et al. found that the intersegmental vessels in zebrafish were actually composed of two endothelial cells, and not one single endothelial cell as previously believed [10]. The molecular mechanism by which multi-cellular cords lumenize was studying in the dorsal aorta of mice and this work also disagreed with the proposed model of intra-endothelial vacuole fusion. The authors showed that lumenization required the polarization of two adjacent

endothelial cells which make up a angioblastic cord [11]. The observed endothelial cell polarization was followed phosphorylation of Myosin Regulatory Light Chain (MLC), which induces contraction actin-myosin fibers such that a lumenal space was created as the two endothelial cells pull away from each other. The authors did not find evidence of large vacuoles in the mouse dorsal aorta and suggested that this space fills through the influx of extracellular fluids that can enter because of the leaky nature of the early blood vessels [11]. The process of lumen formation begins at 2 somites and by 3 somites, fluorescent angiograms results in fluorescence throughout the dorsal aorta and yolk sac blood vessels [3], showing that by this stage extra-embryonic vessels are also lumenized and that a functional circuit for blood flow is present. It is still unclear whether cardiac function is necessary for lumenization. In zebrafish *silent heart* mutants (*sih*), which lack a heartbeat, there is a reduction in vascular lumen formation in the intersegmental vessels and no lumen in the dorsal lateral vein at a stage where lumenization has occurred in the wild-type embryos [12]. Though this would imply that proper lumenization requires flow, the dorsal aorta and yolk sac blood are already lumenized at 3 somites in mouse embryos, meaning that lumenization occurs before the onset of cardiac function [11]. Cardiac induced pressure or flow is likely to enhance lumenization but not be an absolute requirement for a vessel to lumenize.

The formation of a complete circulatory loop is remarkably well timed with the initiation of the cardiac contraction. The first electrical and contractile activities of the heart are observed at 3 somites [2]. These early contractions appear more as small, variable twitches (personal observation, [2, 13]). The pacemaker region is not fixed at 3 somites, causing the location where contraction initiates to change from one contraction to another [2]. Therefore, whether these early contractions represent a true heart beat is disputable. The early cardiomyocytes are, however, already electrically coupled, most likely through gap junctions, such that the action potential can propagate along the heart and create a coordinated contraction [2]. By 4 somites, the strength of contraction and the coordination increase such that a proper heart beat is easily recognizable. The heart rate is low initially (20 beats per minute), but increases as the heart develops [13]. Therefore, though the heart begins to beat as soon as the vessels connect, the early contractions are not believed to be powerful enough to induce circulation of the erythrocytes.

At the stage where the heart begins to beat, the lumenized network of blood vessels contains only blood plasma and erythrocytes are still confined to the blood islands. The origin of embryonic blood plasma is unresolved. Florence Sabin proposed that blood plasma arose from liquefaction of cell within the angioblastic cords in chick embryos [8]. Vacuoles are not visible during lumenization in mouse and the process of lumen formation and by extension plasma formation, may be different. Strilic et al hypothesized that in mouse, interstitial fluid from tissue surrounding a vessel undergoing lumenization created the initial blood plasma [11], but no studies that we are aware of have specifically addressed the origin of embryonic blood plasma.

The blood plasma requires much less force to begin flowing than whole blood and we previously showed that the plasma begins to flow with the onset of cardiac contraction. We used a technique called Fluorescence Recovery After Photobleaching (FRAP) to measure whether plasma flow was present [3]. FRAP involves bleaching a fluorescently labelled sample with a high intensity laser. These bleached fluorophores cannot emit fluorescence and therefore the recovery of a fluorescent signal occurs as new fluorescent molecules move into the bleached region. FRAP was developed to measure diffusion and not flow. The presence of flow causes such a significant increase in the rate of fluorescence recovery. Therefore, if one knows the rate at which fluorescence is recovered in the absence of flow, one can infer the presence of flow if fluorescence is recovered several times faster. We therefore labelled embryonic blood plasma by injecting a fluorescently labelled dextran. We stopped the embryonic heart, bleached regions of blood plasma and measured the rate at which fluorescence was recovered to measure the baseline in pure diffusion. When we then measured fluorescent recovery in embryos with a heartbeat, we observed fluorescence was recovered 10 or more times faster than when a heart beat was present, even in embryos as young as 3 somites. This establishes that plasma begins to flow as soon as the heart begins to beat.

After the onset of plasma flow, the next major event in establishing circulation is the entry of the erythrocytes into circulation. The first erythrocytes can be observed outside the blood islands as early as

5 somites either by staining for βH1-globin [4] or as we did using mice expressing GFP driven by the ε-globin promoter (Figure 1) [3]. Between 5 and 7 somites, only a handful of erythrocytes are present outside of blood islands (arrowheads, Figure 1). Therefore the question arises whether most erythrocytes do not enter circulation immediately because they are adherent to the endothelium or whether plasma flow is simply not strong enough to dislodge them. Work in zebrafish has shown that before the onset of circulation, erythrocytes extend membrane protrusions towards the endothelial cell that stain positive for the focal adhesion protein vinculin [14]. They found that the cleavage of this adhesion between the endothelium and the erythrocytes required metalloprotease MMP8. When they knocked-down MMP8 by morpholino, they found significantly diminished numbers of circulating erythrocytes even though plasma flow was normal. Though their work shows that loss of adhesion is necessary for cells to enter circulation, they stated that they could not establish whether loss of adhesion was a prerequisite or a trigger for the entry of erythrocytes into circulation. Using mouse embryos, we have tested whether erythrocytes are adherent before the onset of flow (Figure 2, collaboration with M.H. Baron, M.E. Dickinson and S.E. Fraser). We used a jet of fluid on the outside of the yolk sac of mouse embryos to attempt to dislodge or push erythrocytes out of the blood islands at stages before they normally enter circulation. At four somites, erythrocytes could not be dislodged by this method. From 6 somites onwards, however, the blood islands could be broken up by externally applied pressure. Since most erythrocytes do not enter circulation until much later, our results suggest that at least in mice, loss of adhesion is a pre-requisite and not a trigger for erythrocytes circulation. The timing of the first erythrocytes exiting the blood islands does, however, coincide with when adhesion appears to be lost. It is still possible that weaker adhesion proteins hold the blood islands in place after 6 somites but that these bonds are not strong enough to prevent external pressure from dislodging the cells. It seems more likely though that the heart is simply not strong enough to induce the circulation of all erythrocytes until later in development.

The entry of erythrocytes occurs stochastically between 5 and 8 somites (Figure 2, in agreement with results from [4]). The first erythrocytes that leave the blood islands do not circulate in the proper sense of the word. From time-lapse microscopy, these cells appear to move with the plasma flow then become

lodged at a more distal location in the vasculature and remain “stuck” there [3]. Three possible explanations exist for this phenomenon. It is possible that remnants of adhesion proteins are present on the erythrocytes such that they re-attach to the endothelium sporadically. Alternatively, it is possible that early plasma flow patterns create points of stagnation in the plasma flow where these first circulating erythrocytes become entrapped. Lastly, vessels lumenization could be heterogeneous such that the diameter of some blood vessels could be too small to allow passage of a red blood cell. As development continues, an increasing number of erythrocytes enter circulation. By approximately 7 somites, some erythrocytes can be observed circulating continuously though the blood islands are still an obvious structure in the yolk sac. The presence of continuously circulating erythrocytes coincides with the onset of cardiac looping (Figure 3). Furthermore, when measuring plasma flow by FRAP at 6-7 somites, fluorescence is recovered in the bleached dye spot at significantly higher rates than in embryos between 3 and 5 somites [15], indicating that much faster plasma flow is present at these stages. This supports the idea that a significant increase in plasma flow occurs preceding the onset of erythrocyte circulation. The blood islands disperse by around 11 somites, when all the erythrocytes in the yolk sac have entered circulation. The hematocrit, or volume percent of the blood occupied by red blood cells, can be measured by a line scanning technique we previously developed [15]. Using this technique, we observed a rapid increase in the hematocrit after 7 somites, with the hematocrit reaching a steady level by 10-11 somites when the blood islands have dispersed (Figure 4).

3. Shear Stress In Vascular Development

As red blood cells enter circulation, endothelial cells are exposed to a new physiological signal, shear stress. The heartbeat also creates blood pressure that in turns creates circumferential stretch on the blood vessel wall. Though circumferential stretch is likely to be important for vascular development, very little research has investigated the role of pressure during cardiovascular development and this review will therefore focus on shear stress. Shear stress is a frictional force that is created by a flowing liquid. A stress simply means a force per unit area. A normal stress is one acting perpendicular to a surface and a

shear stress only implies that the force is tangential to the surface in question. Shear stress is created from the friction of the fluid molecules interacting with the wall of the blood vessels. Endothelial cells line the blood vessel wall and endothelial cells can sense this force through a process known as mechanotransduction. Endothelial cells are capable of sensing shear stress by many different mechanism including shear-activated ion channels [16], plasma membrane fluidity [17], cytoskeletal deformation [16] and primary cilia [18]. Primary cilia are found only in regions of low shear stress [19, 20] and may be of particular importance during vascular development since the levels of shear stress are much lower than in the adult [15].

The measure of a fluid's resistance to flow is called the viscosity of the fluid. Since shear stress arises from friction between particles in motion and the wall, both the density of particles and the nature of the molecules (size, charge, etc) affect a fluid's viscosity. Liquids that resist flow such as honey have high viscosity whereas a gas would have a very low viscosity. The viscosity of blood flow is heavily dependent on the concentration of red blood cells. Shear stress, which is calculated by multiplying the velocity gradient of flow at the wall by the viscosity, would be very low in the absence of flowing erythrocytes, below levels that endothelial cells are believed to be capable of sensing. Therefore the entry of erythrocytes into circulation represents the point in vascular development where physiological signals from shear stress would first be recognizable by endothelial cells.

The first blood flow present during development is very different than normal blood flow patterns in the adult. Early blood flow in the embryo shows flow reversal, such that during systole blood is flowing down the vessels but during diastole blood flow backwards. Movie 1 illustrates blood flow in a quail embryo at 13 somites, just after the onset of blood flow. The flow has been seeded with 1 μm fluorescent beads and is imaged at 500 frames per second and is replayed at half speed. Blood flows from the heart, descends through the dorsal aorta (DA), to the yolk sac and then returns to the heart (Hrt, Figure 5, Movie 2). Flow is pushed to distal regions of the yolk sac simply because the vessels are small and offer significant resistance to large volumes of blood flow. Arterial and venous regions at this point are spatially separate and already express different markers of arterio-venous identity such as ephrinB2 and EphB4 [21, 22].

Pulsatility is found in all vessels at this stage including arteries and veins. In the adult, only arteries experience pulsatility and flow is steady in veins. Shear stress levels are also much lower during development than in the adult. Typical levels of shear stress in an adult mouse are around 50 dyn/cm^2 [23], whereas the maximum shear stress in E8.5 mouse embryos is believe to be around 5 dyn/cm^2 [15].

Thoma was the first to suggest that fluid dynamics affected vascular development [24]. He theorized that during vascular development, the vessels which carried the largest amount of flow enlarged and those which carried the least blood flow regressed. Blood flow not only creates mechanical forces but also brings oxygen and nutrients to tissues. To differentiate between mechanical forces created by blood flow and transport of chemical factors, we used the fact that shear stress is dependent on the viscosity of the fluid and that blood viscosity is mainly determined by the concentration of erythrocytes [3]. Since erythrocytes remain confined to the blood islands early in vascular development, we injected a polymer matrix into the blood islands to prevent the erythrocytes from entering circulation. In this situation, vascular development stopped at the capillary plexus and vascular remodelling did not occur. Therefore, the circulation of erythrocytes is necessary for vascular remodelling to occur. Since erythrocytes also carry oxygen, we needed to separate the role of erythrocytes as oxygen carriers from their role in increasing viscosity. We therefore injected a starch solution into embryos in which we had sequestered the erythrocytes such that we produced a viscous flow in the absence of red blood cells. This rescued vascular remodelling as well as the expression of normal shear-induced genes such as endothelial nitric oxide synthase (eNOS) [3].

Blood flow and associated mechanotransduced signals have recently been connected to the production of hematopoietic stem cells (HSCs) during development. North et al. used a chemical screen in zebrafish to identify regulators of HSC formation in the aorta–gonad–mesonephros (AGM) [25]. They observed that many of the chemicals that effected HSCs formation were modulators of either the heartbeat or blood flow. They also found that zebrafish mutants which lacked blood flow, such as *sih* mutants, had reduced number of HSCs and that these defects in haematopoiesis could be rescued by nitric oxide donors. North et al. proposed that blood flow induced nitric oxide production that in turn enhanced HSCs

production of the AGM [25]. Blood formation can, however, occur *in vitro* from isolated explants of the AGM showing that blood flow may enhance blood forming ability but is not a requirement for HSC formation [26]. Cardiac valve formation and the onset of unidirectional flow coincide with the time when HSCs begin to form in the AGM. It would therefore be interesting to look at whether HSCs formation is reduced or delayed in mice with abnormal cardiac valve formation which fail to produce unidirectional flow, such as the NFATC$^{-/-}$ mice [27]. Since the level of shear stress is directly affected by the concentration of erythrocytes in blood, shear stress induced HSC production represents a positive feedback mechanism whereby shear stress creates more blood cells, which in turn increases viscosity and thereby increases the level of shear stress. The total hematocrit is largely composed of erythrocytes and not HSCs. Therefore, shear-induced HSCs production is unlikely to produce significant changes in the overall blood viscosity.

Evidence has also supported a role for hemodynamics in vascular remodelling that occurs later in development, such as cardiac cushion formation [28] and aortic arch artery development [29]. The aortic arch forms by regression of select branchial arch arteries. This regression is initially symmetric, however the sixth branchial arch artery regresses asymmetrically. Using echocardiography, Yashiro et al. showed that flow specifically increased in the left branchial arch artery over the right branchial arch artery during remodelling of this system and that in Pitx2$^{\mathbf{\Delta}\text{ASE}/\mathbf{\Delta}\text{ASE}}$ mice, which exhibited randomized laterality of the aortic arch, this increase in flow on the left hand side did not occur [29]. Furthermore, the group showed that they could inverse the lateralization regression of the sixth branchial arch artery by arresting the flow through the left branchial aortic artery. Hogers et al used a venous clip to change blood flow patterns in the chick embryo heart over a period of 5 or more days and found that approximately 50% of embryos showed abnormal cardiac valve formation (either semilunar or AV) and 50% of embryos showed abnormal aortic arch formation [30].

4. Shear Induced Genes During Vascular Development

In the adult, shear stress is a potent activator of many genes. The type of flow which is present is an important determinant of which genes are activated (for review, see [31]). As an example, the expression of genes involved in arterial differentiation can be induced in endothelial cells *in vitro* by exposure to pulsatile flow but not steady flow. Similarly, venous genes are upregulated when cells are exposed to steady flow but not pulsatile flow [32]. Steady laminar shear stress is generally considered cardioprotective. Oscillations and recirculation in flow are associated with diseases, such as atherosclerosis. As mentioned, blood flow in the embryos shows flow reversal during the cardiac cycle, which is never present in a healthy adult vasculature. Furthermore, branching patterns in the embryo are abnormal leading to local eddies in the flow [15]. The embryonic vasculature is also unable to dynamically regulate the lumenal diameter of vessels when flow first begins because smooth muscle cells have not yet differentiated. Therefore, shear induced gene expression patterns are likely to be quite different during vascular development than in the adult.

One of the most well studied flow-induced effects on gene expression in the adult is the upregulation of endothelial nitric oxide synthase (eNOS) and flow-induced production of nitric oxide. When blood flow increases in the adult, shear stress induces an upregulation of eNOS leading to the production of nitric oxide, a vasodilator. Vessel dilation causes the velocity of blood flow through the vessel to decrease and this normalizes the stress. Nitric oxide is also involved in remodelling due to chronic changes in blood flow, playing an important part in upregulating angiogenesis and in mediating leukocyte requirement. During development, however, the role of nitric oxide is not well understood. Mice in which all three nitric oxides are ablated are viable [33] and therefore it was believed that nitric oxide production was not essential for early vascular development. Ablation of eNOS does cause defects in later vascular development, leading to congenital vascular defects such as ventricular septal defects [34] and abnormal pulmonary vascular development [35]. Some recent evidence has come out supporting a role for eNOS in early vascular development however. It was shown by ultrasound that within a given litter, gestation loses are significantly higher for eNOS-/- litters than for wild-type litters [36]. The most common stage at which embryos died was between E8.5 and E10.5 which is also the stage at which vascular remodelling is occurring. If mice embryos are cultured in the presence of NOS inhibitor L-NMMA between E8.0 and

E9.0, vascular remodelling fails to occur [37]. eNOS expression at mid-gestation occurs only after flow is established [38] and this expression by can attenuated by lowering shear stress [3]. Therefore, though embryos in which nitric oxide synthases have been ablated are capable of vascular remodelling, the role of flow induced NOS may be more subtle, possibly created positive feedback loops which enhance but are not necessary for vascular development as is seen during hematopoietic development [25].

Another hallmark of mechanotransduction is the upregulation of Kruppel-like factor 2 (KLF2). In fact, KLF2 appears to be upstream of many mechanotransduced genes. *In vitro*, endothelial cells in which KLF2 has been knocked down fail to upregulate genes such as endothelin-1 and eNOS when exposed to laminar flow [39]. During development, KLF2 expression in the heart is highest in regions that should be exposed to the fastest flow. Deletion of KLF2 in a mouse model causes increased cardiac output during development that eventually leads to congestive heart failure. This increase in cardiac output is believed to be caused by a lack of vascular tone since administration of phenylephrine, which would increases vascular tone, can partially rescue vascular development in these embryos [39]. Since KLF2 expression appears to be required for the expression of many flow-induced genes, embryos that lack KLF2 present themselves as an important tool for dissecting the molecular mechanism by which flow-induced signals can affect vascular development.

5. Conclusions

Though it is clear that the physical forces created by the onset of blood flow are biologically active in the embryo, understanding how these forces affect vascular remodeling and gene expression is still unclear. Genetics play an important role in vascular development, but gene expression is affected by the local flow dynamics. The vascular network and the embryo cannot grow without proper blood flow. For these reasons, understanding the process by which flow initiates and the signals which it provides are of primary importance to the study of developmental vascular biology.

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Table I – Summary of Early Mouse Cardiovascular Development

Mouse Developmental Stage	Cardiovascular Development
0-1 somites	- Expansion angioblasts from blood islands - Angioblast differentiation in embryo proper
2 somites	- Onset of lumenization of the dorsal aortae
3 somites	- First contractions of cardiomyocytes - Vascular network formed and lumenized - Onset of plasma flow
5 somites	- Loss of erythroblast adhesion in blood islands - First erythroblasts outside of blood islands
7 somites	- Initiation of heart looping - First circulating erythroblasts
11 somites	- Blood islands disperse

Figure Legends

Figure 1 – Initiation of Blood Flow. Freshly dissected ε-globin::eGFP mice were used to assess the initiation of blood flow. Embryos were dissected in heated media to ensure normal heart function. The first erythrocytes are observed outside the blood islands by approximately 5 somites. These cells do not flow in the proper sense of the word but move with plasma flow for a short time before becoming immobilized at a secondary location. By 7 somites, the first continuously circulating erythrocytes can be observed. Flow is observed throughout the yolk sac by approximately 8-9 somites. These results are in agreement with previously published results [4].

Figure 2 – Mechanical Disruption of the Blood Islands Indicates that Adhesion is Lost in Mouse Embryos Between 4 and 6 somites. The adhesion of erythrocytes within the blood islands was investigated during early vascular development. The blood islands were exposed to a jet of pressurized liquid on the outside of the yolk sac in an attempt to dislodge erythrocytes at stages where they would normally remain confined to the blood islands. The blood islands were observed using a transgenic mouse in which eGFP expression is driven by the ε-globin promoter [40]. At four somites, external pressure could not successfully dislodge the erythrocytes. From 6 somites onwards, it was possible to disperse erythrocytes using external pressure, red arrowheads.

Figure 3 – Cardiac Looping Begins at 8 Somites in Mouse Embryos. The embryonic heart is a linear tube until 7 somites in mouse embryos. The heart then undergoes looping, the first stage in the formation of a four-chambered heart. Images show the morphological structures present at various stages, highlighting cardiac shape, future atrial (A) and ventricular (V) regions as well as the direction of blood flow (blue arrows). Looping begins at 7 somites, which coincides with the stage at which erythrocytes begin to circulate continuously.

Figure 4 - Hematocrit with Respect to Somite Stage. Hematocrit, or the volume percent of erythrocytes in whole blood, was plotted with respect to somite stage for mouse embryos. Continuous

circulation is first observed at 7-8 somites. At these early stages, most erythrocytes remain confined in the blood islands and the overall hematocrit is low. As the blood islands disperse, the hematocrit increases rapidly reaching steady levels of approximately 35 vol% by 10 somites.

Figure 5 – Initial Pattern of Blood Flow During Embryonic Development. Blood flow pattern in a 12 somite chicken embryo. The blood is pumped by the heart (Hrt) through the dorsal aortae (DA) into the capillary plexus of the yolk sac. The blood flow in the capillary plexus goes both by proximal and distal routes (arrows, see also Movie 2). At this stage, caudal regions of the yolk sac are arterial and rostral regions are venous. Hd; head.

Movie 1 – Flow Reversal is Present When Heart First Begins to Beat. Embryonic blood flow in a 13 somite quail embryo shows retrograde flow during diastole. Blood flow has been seeded with 1 μm fluorescent particles and imaged at 500 fps with a 10x objective lens.

Movie 2 – Blood Flow in the Capillary Plexus of a 17 Somite Quail Embryo. Blood flows from dorsal aorta to both proximal and distal regions of the capillary plexus. Blood flow has been seeded with 1 μm fluorescent particles and imaged at 500 fps with a 5x objective lens.

Figure 1
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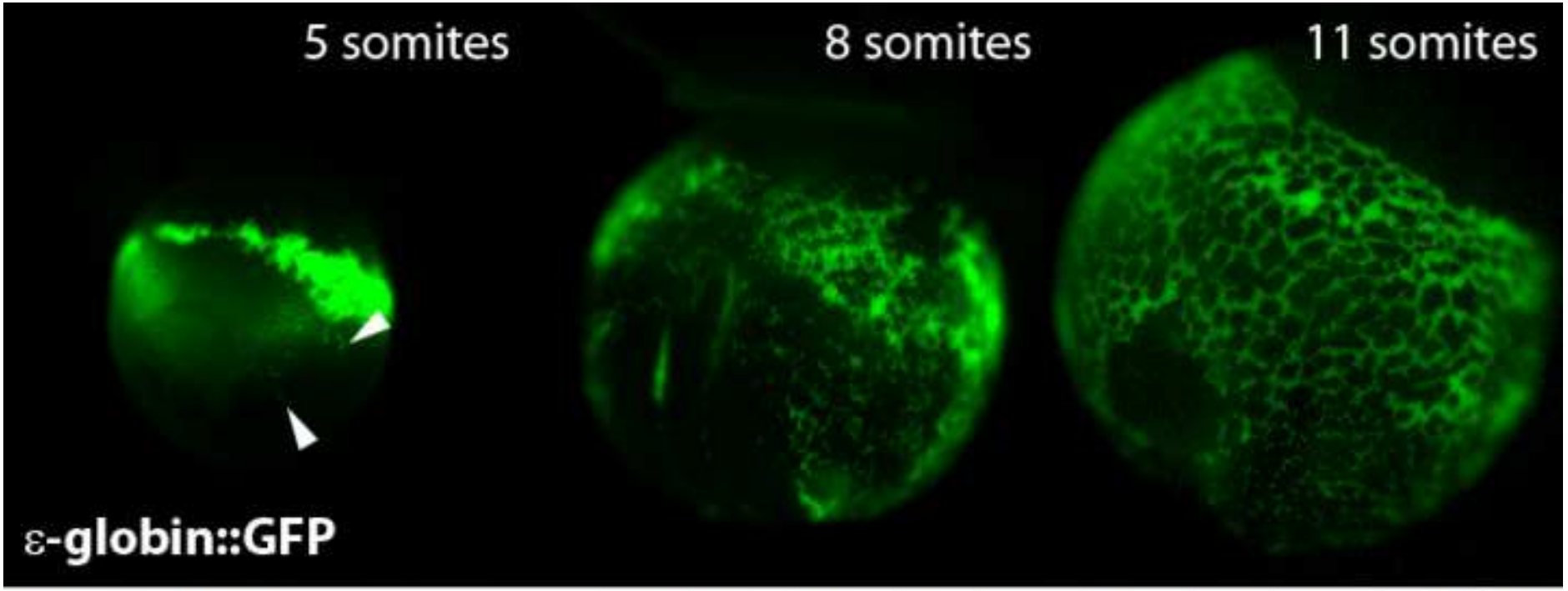

Somite stage	0-2	3	4	5	6	7	8	9	10	11
Blood Islands Only	6	3	3	3						
Some expansion proximal to blood islands			1	2	2					
Extensive blood island expansion				3	4	1				
Some flow, in distal yolk sac						8	5	3	1	
Full flow present							3	5	6	4
Total (number of embryos observed)	6	3	4	8	6	9	8	8	7	4

Figure 2

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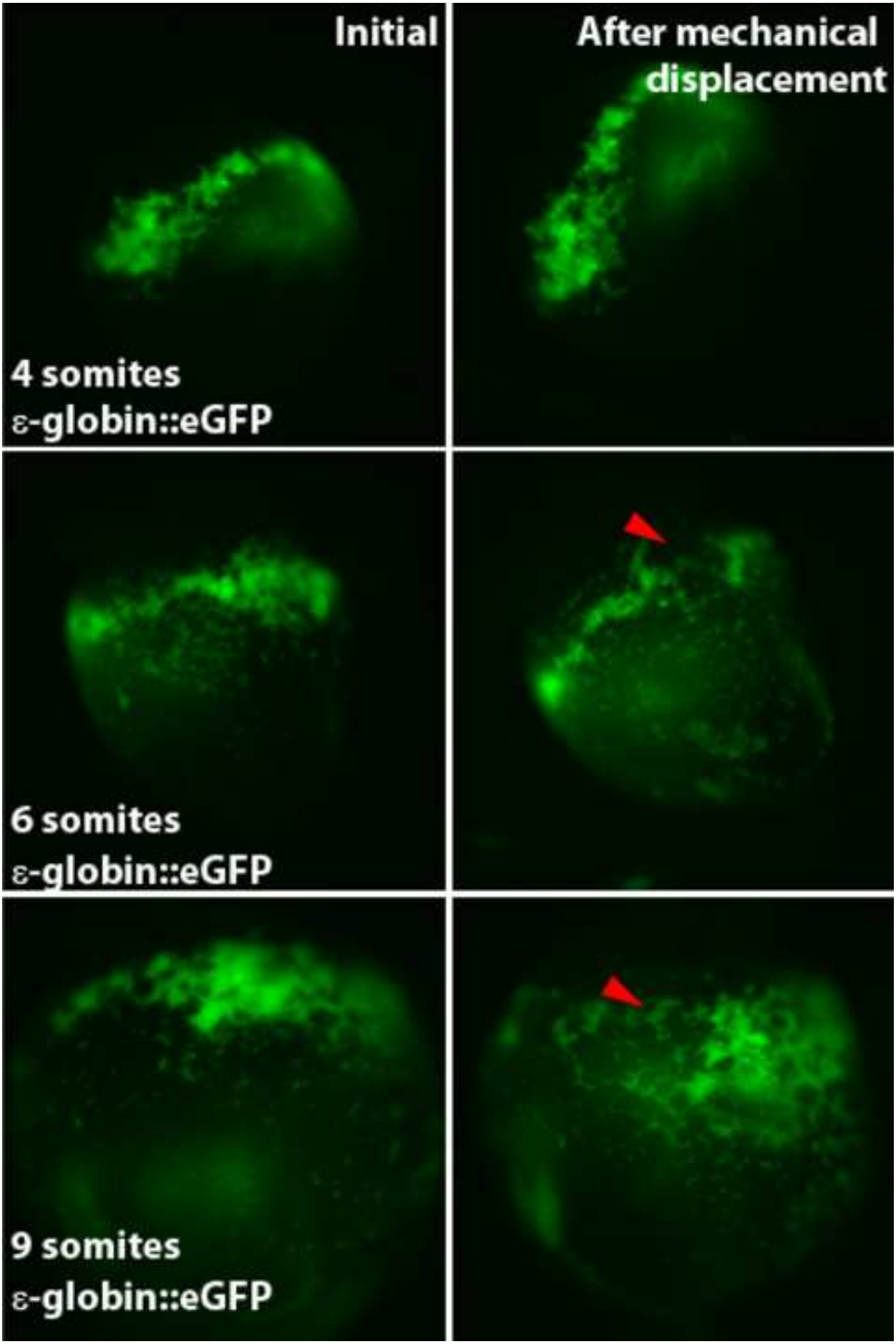

Figure 3
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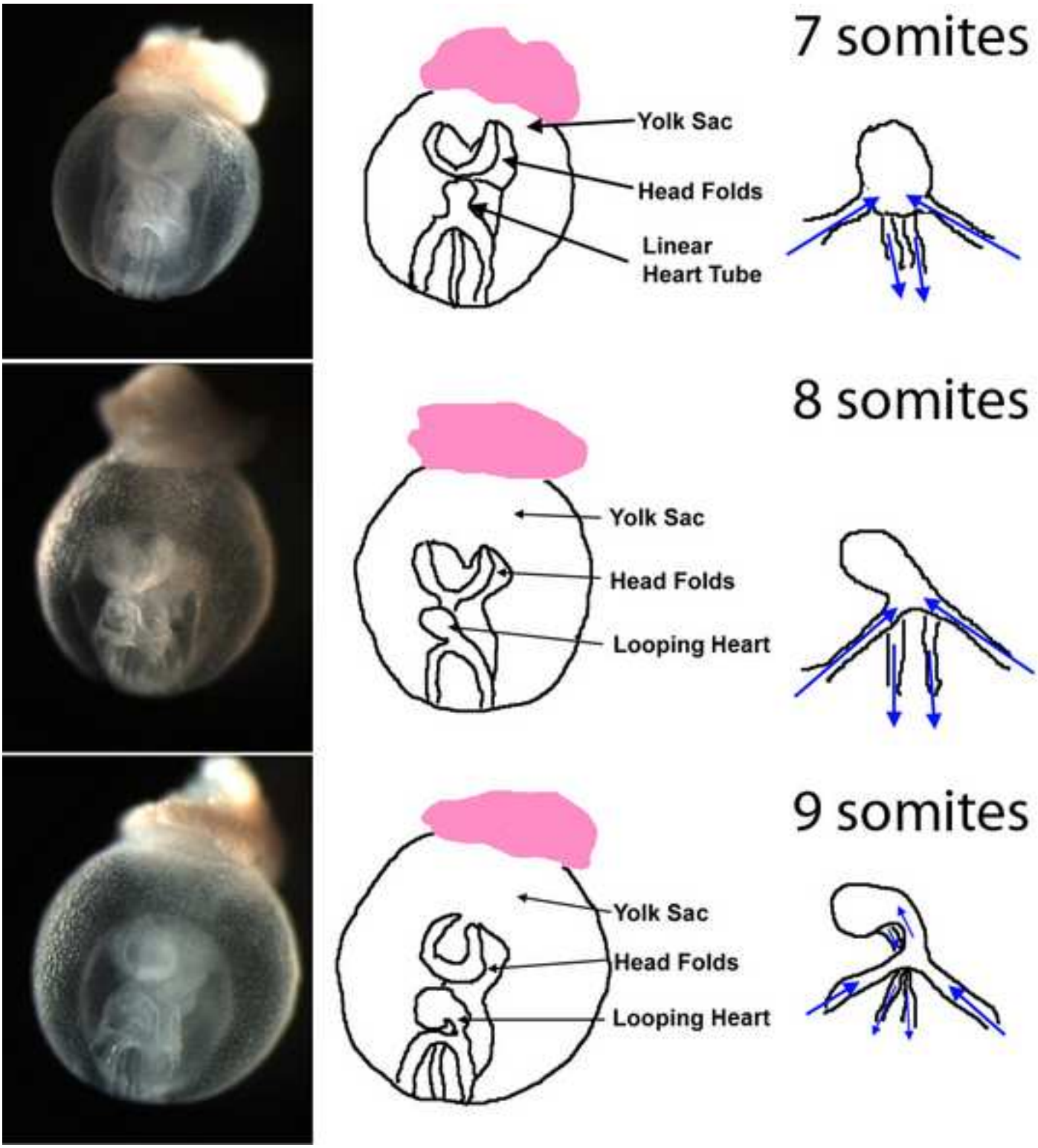

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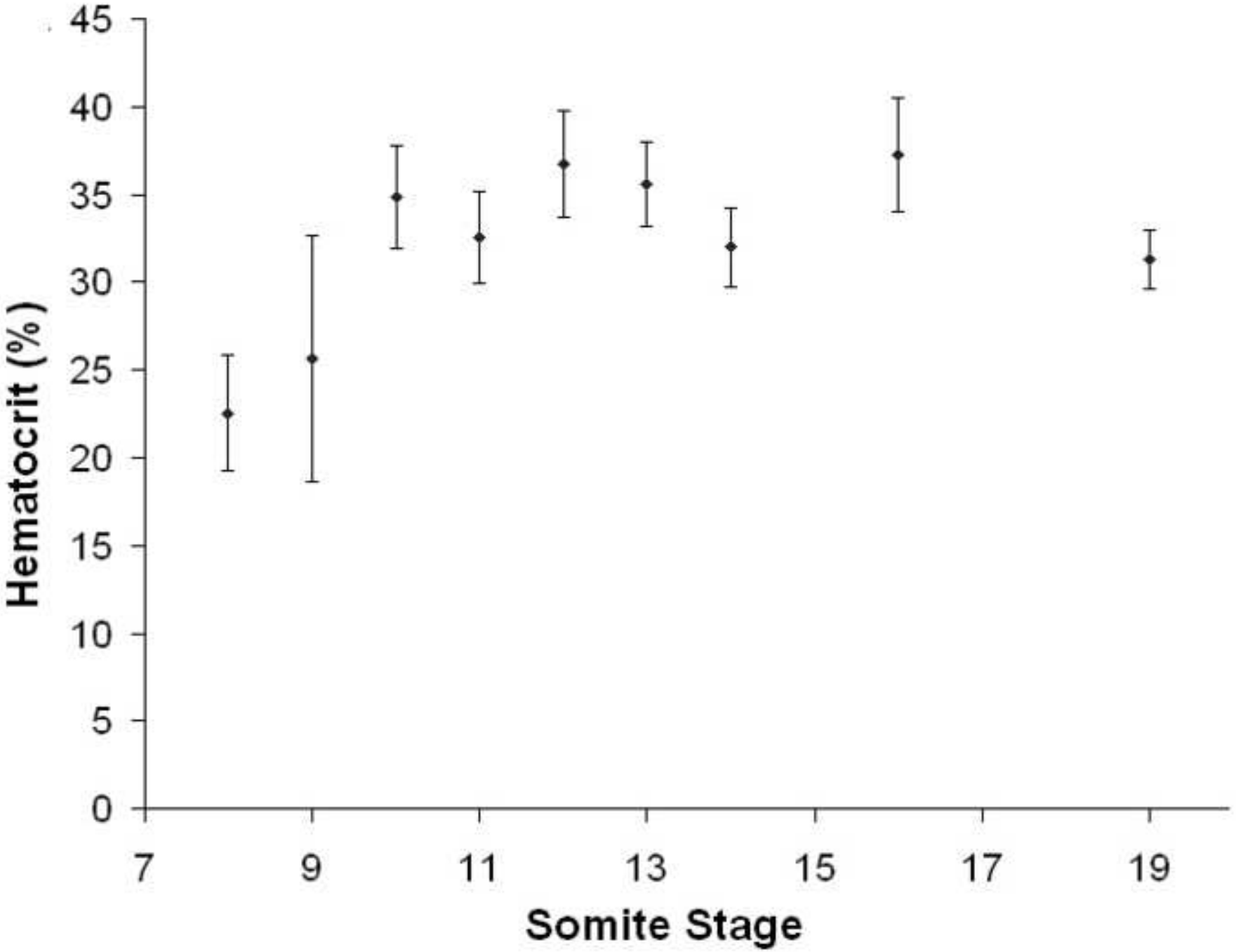

Figure 5

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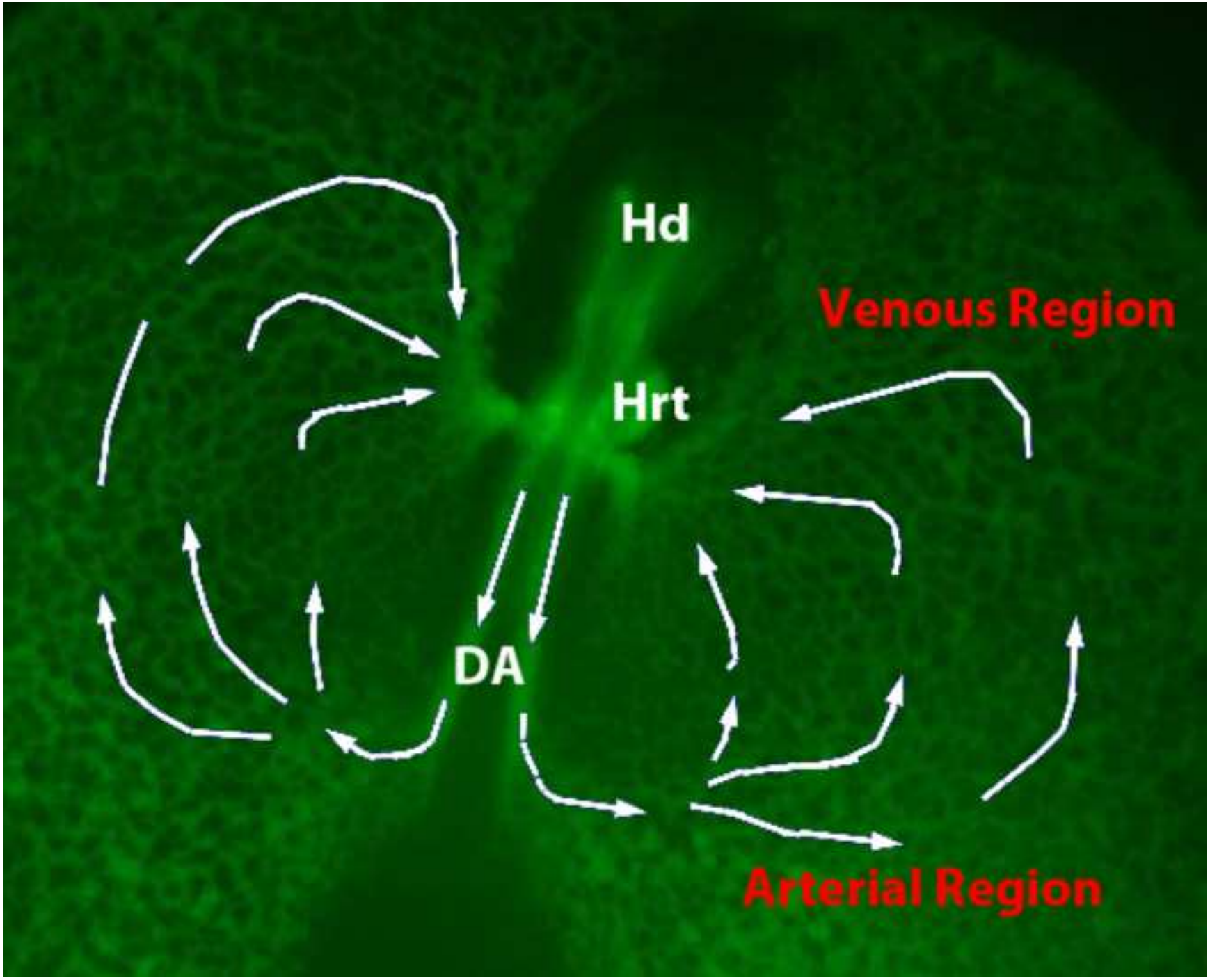

Movie 1

Click here to download Supplementary Material: Movie1 10x.avi

Movie 2

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