

Mechanisms for lesion expansion after microinfarction: in vivo multiphoton microscopy of beating heart

Understanding the microscopic dynamics of the beating heart has been very difficult because the technical challenges of imaging with micrometer resolution while the heart moves have been prohibitive. Recently, the development of multiphoton microscopy has made possible in vivo studies of function and failure in brain and other motionless organs, resulting a new appreciation of how systemic and circulatory influences affect local function¹. In heart, studies of in vivo cellular dynamics and blood flow at the cell and capillary level are therefore far behind other organ systems. We have recently piloted the ability to measure microscopic phenomena in the beating heart and now have the capability within a live rodent to image single myocyte action potentials and contractility as well as capillary blood flow. Recently, coronary microembolization has been recognized as a cause of contractile dysfunction², so we adapt a laser-based microstroke model we developed for brain to coronary arteries as a novel model of myocardial microinfarction³. With these two optical technologies we propose to investigate **how microinfarcts caused by a coronary artery occlusion expand over time and cause contractile deficits in the surrounding cells**. We propose to investigate the mechanisms, now addressable with our novel imaging and lesioning tools, involved in expansion of damage in the first hours after the initiation of a microinfarct.

Aim 1 Determine the type of vascular occlusion that causes myocardial microinfarction.
Objective - identify the type (e.g. epicardial versus penetrating) and characteristics (e.g. diameter and blood flow at baseline) of occluded arteriole that mimics our current understanding of the progression of a small human myocardial infarction in space and time. Laser occlusions enable the systematic study of microinfarcts because they allow the precise occlusion of a single arteriole with functional measurements both before and after the lesion³⁻⁶. Bringing this technology to the heart will enable new studies of coronary disease. Hypothesis - blocking a terminal arteriole would generate an ischemic territory just in the area primarily served by that arteriole because there is no redundancy in the arteriole supply at the penetrating arteriole level. Methods - to match up to existing models, we propose to use established technologies such as ultrasound imaging of wall thickness and perfusion and electrocardiography (ECG). Triphenyl tetrazolium chloride (TTC) staining and histology will generate a time sequence of tissue damage that can be compared to clinical literature.

Aim 2 Identify the microvascular dynamics that contribute to lesion expansion over time.
Objective – look for progressive failure in perfusion at the capillary level that exacerbates ischemia. Mechanisms could include inflammation and vasoconstriction. Hypotheses - first, the accumulation of inflammatory cells in the capillaries around the lesion could slow capillary flow. Second, in addition to direct ischemic damage to the endothelium, inflammatory signaling could lead to endothelium-driven vasoconstriction. Methods - we use multiphoton microscopy to measure red blood cell (RBC) motion and lumen diameter in single capillaries, arterioles and venules before and over time after a vascular lesion. We use high-resolution image stacks in animals with fluorescently-labeled leukocytes to identify whether capillaries are plugged. To confirm mechanism, we interfere with leukocyte-endothelium and pharmacologically inhibit smooth muscle contraction.

Aim 3 Investigate the progression of excitation-contractility dysfunction. Objective – measure severity and distribution of excitation and contraction changes over time. In individual myocytes, we measure contractility and action potentials with calcium imaging before and over time after the lesion to investigate whether excitation or contraction dysfunction spreads. Hypotheses - the region of dysfunction is driven by expanding blood flow deficits. If so, we expect that the dysfunctional region will expand with similar dynamics as the region of

compromised blood flow. Alternatively, contractile dysfunction may progress without blood flow decrease. Methods - in mice expressing fluorescent calcium-reporting proteins (G-CaMP) we can track the edges of individual myocytes to generate a direct measurement of how much each cell contracts in a heart cycle. The G-CaMP also reports action potential propagation, which will be correlated to contractility in each cell and to the dynamics of blood flow.

B. BACKGROUND AND SIGNIFICANCE

The significance of this proposal for the research community is in the development of new capabilities for measurements of microscopic cardiac blood flow and functionality in the whole animal. The tools and the proposed work could have a great influence on the clinical community because the impact of microscopic lesions may have been underestimated. Understanding how these lesions alter function could identify targets for future therapies.

Unknown impact and progression of cardiac microinfarctions. In brain, improved clinical imaging capabilities led to studies that showed a correlation between microscopic brain lesions and impaired cognitive function⁷. Similarly, it is likely that the importance of microinfarcts for cardiac health has long been underestimated. Occlusions of microvessels caused by emboli, often from atherosclerotic plaques or increasingly common percutaneous interventions, lead to small infarcts that can contribute to heart disease through arrhythmias and contractile deficits². This has been difficult to study in animal models because there are challenges in making microscopic vascular lesions and characterizing their effect. Occlusions in small vessels have been modeled by injections of emboli, but these models must contend with the random location of the occlusion, requiring 3-D imaging such as MRI or exhaustive histological survey of post-mortem tissue ^{8, 9}, greatly limiting the utility of the method for systematic studies of microscopic effects. Unlike larger cardiac lesions, microinfarcts may be clinically silent so would require different strategies than acute heart attacks for treatment, such as prophylactic strategies. In addition, inflammation, and therefore likely leukocyte invasion, are strong modulator of cardiac function^{8, 10}.

Infarction and dysfunction increase over time after occlusion. After ischemia caused by coronary artery occlusion there is a progression in cell dysfunction and damage. It is currently thought that myocardial damage expands from a region in the subendocardium with a gradient of pathology severity from the middle to the edges^{10, 11}. In the region with the most severe blood flow deficit (often at the subendocardium) irreversible damage leads to necrosis beginning about 20 minutes after occlusion¹². Just outside of the necrotic area blood flow and contractility are both reduced¹³. This region may share characteristics with “hibernating myocardium” in that the coupling between contractility and blood flow is preserved, with both decreasing. In the chronic case, it is thought that the drop in blood flow is actually a consequence, not a cause of reduced contractility¹⁰. In addition, glucose utilization increases, perhaps also a protective mechanism. In both the acute and chronic case, an important clinical consequence is the possibility that this region may be returned to normal contractility by bringing blood flow back up to normal values¹⁴.

Further away from the necrosis, the blood flow is near normal, however, contractility is often compromised, often termed “stunned myocardium.”. This is mostly seen in reperfusion where the release of an occlusion or stenosis that brings flow up to normal levels fails to restore contractility, but it is also thought to occur at the border of an ischemic region. Several studies have found contractile deficits that are disproportionately severe relative to the size of the actual infarcted tissue, suggesting that the tissue around the lesions is “stunned”^{10, 11, 14}. Likely candidates for the cause of the uncoupling are changes in calcium responsiveness of myofibrils, calcium handling, action potential propagation, and also interference with contractile

mechanisms by reactive oxygen species¹⁴⁻¹⁶. The surround or edges of an infarct are particularly relevant for the patient, because this represents tissue that is potentially salvageable and also could contribute to normal function later.

Studying microscopic phenomena behind cardiac dysfunction requires new tools. Because it has been difficult to resolve features as small as cells in a moving heart, it has been difficult to study heart disease at the microscopic scale. We tend to conceptualize regions of “stunned” or “hibernating” as concentric volumes around the infarct, but in actual myocardial infarction it is likely that there is a heterogeneous mix of different degrees of damage resulting in cell by cell variations in forces, action potential conduction, and local blood flow. Previous studies have had to eliminate motion or dissociate the tissue in order resolve cells, so that the interaction of the motion and forces on cells within the tissue has been difficult to study^{17, 18}. These preparations also cannot be used to study blood flow.

Bringing *in vivo*, optical, cell-resolved imaging to the heart. The first of two new optical tools for cardiac research is multiphoton microscopy, which provides the capacity to image fluorescence with micrometer resolution deep within a living sample. This microscopy, also called two-photon microscopy, can be used for quantitative measurement of single-cell dynamics in an intact organism. Multiphoton microscopy has been used to study the dynamic interactions among different types of cells in neurological disease^{19, 20}, as well as to monitor the activity of excitable cells in the brain via indicator dyes²¹. It is a unique tool for quantifying cellular behavior within the complex environment of the living tissue. Optical contrast in multiphoton microscopy relies on achieving a sufficiently high laser intensity so that multiple photons will simultaneous interact with a fluorescent molecule to excite it. Such photon density is only achieved at the focus of a femtosecond laser, so the nonlinear optical signal (e.g. two-photon excited fluorescence or second harmonic generation) is only generated in the micrometer-sized volume of the laser focus. The strength of the nonlinear signal is then measured as a function of the three-dimensional position of the laser focus inside the material to build a three-dimensional map. Importantly, the signal photons do not have to be imaged, only detected, so that even scattered fluorescence photons can contribute to the detected signal²². This enables multiphoton microscopy to “optically section” and resolve an approximately micrometer thick plane deep within a scattering sample. In brain, we have been able to image capillaries as deep as 1 mm²³.

Challenges in measuring cardiac tissue blood flow. The motion of beating heart is necessary to drive blood flow, but at the same time causes so much movement that multiphoton microscopy has not been practical. Coronary perfusion has been difficult to measure in part because measurement techniques can affect the heart pumping and change the coronary perfusion. For example, the injection of tracers that are retained in the coronary tissue such as beads or radiolabeled molecules can be used to estimate perfusion but requires euthanasia and cannot be used to measure dynamics^{24, 25}. Imaging modalities such as PET, MRI and ultrasound can also be used to infer blood flow, but are limited in spatial resolution. Although multiphoton and other types of microscopy have been used on isolated cardiac cells or tissue, these strategies cannot be used to study blood flow because they necessitate paralyzing the cardiac motion²⁶. Studies of perfusion in brain have benefitted greatly from multiphoton microscopy that enable the quantification of blood flow on the single microvessel level by enabling studies of the heterogeneity and fluctuations of flow^{27, 28}, the mechanism for neurovascular coupling²⁹ (the brain’s redirection of blood flow to regions of active neurons with ~100µm resolution), and the impact of microvascular occlusions³⁰. In addition to the velocity of red blood cells, multiphoton microscopy enables the additional measurements such as identification of motion of leukocytes and quantification of the diameter of vessels so that the

impact of processes such as inflammation and vascular regulation on local tissue perfusion can be studied^{5, 20}.

Vascular dysfunction due to ischemia. Endothelial cells lining the lumen of vasculature regulate vessel tone. These cells are highly sensitive to damage during reduced blood flow, so that an occlusion can cause dysregulation of smooth muscle and increased vascular contractility³¹. Ischemia can spread due to vasospasm triggered by signals from the damaged endothelium. For example, increased extracellular potassium can convert what is normally a dilatory signal into a contractile signal³². In brain, the region of stroke can expand during waves of depolarization which also cause a wave of vasospasm³³. In addition, damaged endothelial cells and smooth muscle cells can change in morphology and can interfere with blood flow by “ruffling” and protruding into the lumen of vessels. Such effects are particularly potent in the capillaries where RBC and leukocytes must already deform to push through a normal vessel. A slight obstruction of the lumen can change the resistance of the capillary drastically³⁴. By-products of ischemia such as the extracellular potassium and cytokines can diffuse from the origin, impacting previously undamaged vasculature. Cytokines and inflammatory signals from the myocardial cells that have been damaged by ischemia will also trigger the recruitment of blood-borne inflammatory cells. Although flow in the territory of the occluded vessel is low, collateral flow is maintained even without reperfusion³⁵ and can serve as a conduit for leukocytes. On a short time scale, most of the inflammatory cells are likely neutrophils³⁶. Especially in the smaller vasculature such as capillaries, a leukocyte adhering to activated endothelium can plug the capillary, effectively occluding the vessel and comprising flow³⁷.

C. PRELIMINARY STUDIES

Collateral blood flow after occlusions depends on redundancy of vascular network. In brain, our studies of cortical microstroke have generated a good understanding of the vascular redundancy and blood flow delivery network capacity^{3, 4}. Because the coronary vascular architecture nearly exactly reflects the vascularization of the brain cortex, insights from brain can inform expectations for the heart. In both, the vascular supply and drainage is provided by a network of communicating arterioles and venules which are bridged to the capillary network by penetrating, terminal arterioles and ascending venules^{38, 39} (Fig. 1b). Similar to heart, it is thought that performance of brain tissue is metabolically limited so that function (neural activity) regulates blood supply (neurovascular coupling). Also like heart, the brain has no energy reserves and lesions caused by occlusions of blood vessels exhibit progressive lesion expansion over time. To induce clot formation in a blood vessel, we use optical techniques developed in brain to locally damage the endothelial wall of the vessel, triggering the natural blood clotting cascade. The injury is induced using photodisruption³, where an intense femtosecond laser pulse is absorbed in the vessel. The absorption is nonlinear, meaning that multiple photons must simultaneously interact with the same molecule, a process that can only happen at the focus of a high intensity laser pulse. This enables the occlusion of the targeted vessel without disruption of the tissue or other vessels, above or below the target (Fig. 1a). The location of a clot in the vascular hierarchy critically influences how the flow in neighboring vessels changes (Fig. 1c). For example, the surface communicating arterioles show much greater fault-tolerance than the downstream microvascular network (Fig. 1c). In agreement with these dynamical findings, the topology of the communicating arteriole network is more redundant than the sub-surface capillary network. The greatest degree of ischemia is produced by clotting the penetrating arterioles (Fig. 1c), where there is the least amount of redundancy.

Mechanical stabilization of heart and ECG-gating enable imaging. We devised a combination of surgical methods and image acquisition strategies to modify our custom-built

multiphoton microscope for imaging in rodent heart *in vivo*. Our surgical method physically restricts the motion of a small part of the heart, while enabling most of the heart to beat freely (Fig. 2). We anesthetized mice with isoflurane and ketamine-xylazine and placed them on a metal surgical platform with a heating pad. Animals were artificially ventilated and the chest cavity was opened. A customized silicone-coated retractor was placed between heart and lungs to isolate the heart from respiratory motion. We designed a support system with a glass window in a metal frame to allow optical access to the heart. We use a reconstituted fibrin-thrombin solution to adhere a small portion of the heart (~2-3 mm diameter) to the glass window to minimize motion artifacts (Fig. 2). The ECG was acquired with subcutaneous electrodes inserted in the left front and right rear paws. We image using a custom-designed multiphoton microscope with a 20x water-immersion objective (0.95 numerical aperture; Olympus) and use 800-nm, 87-MHz, 100-fs pulses from a Ti:sapphire laser oscillator (MIRA HP; Coherent) or 1043-nm wavelength, 1-MHz, 300-fs pulses from a fiber laser (FCPA μJewel D-400; IMRA). We synchronize image acquisition to the ECG to further minimize motion artifact, so that frames were acquired during the same phase of the cardiac cycle.

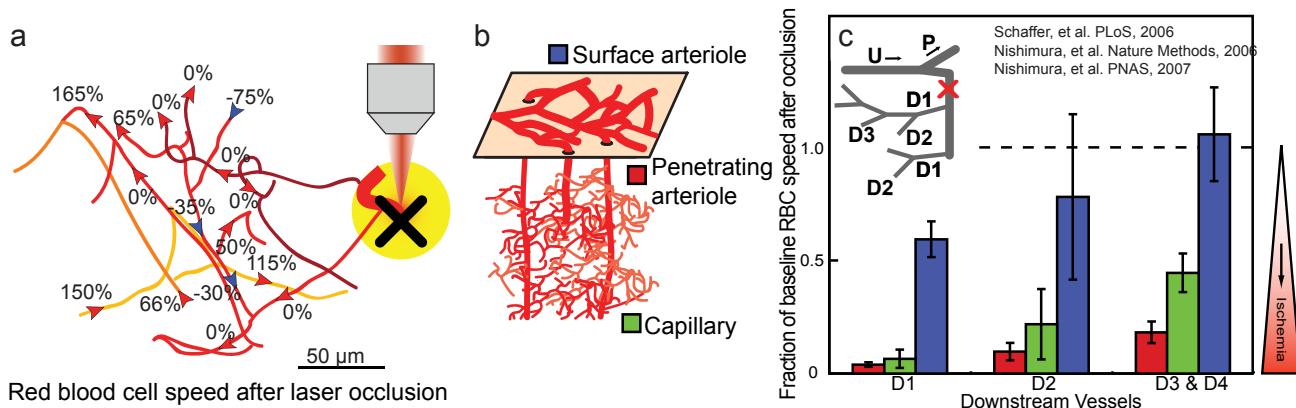


Figure 1 Post-occlusion blood flow depends on vessel topology. (a) Schematic of capillaries about 150 μm below surface showing red blood cell speed as a fraction of baseline in brain. X marks a penetrating arteriole that was occluded with laser ablation. (b) Brain and coronary vasculature have surface vessels which branch to penetrating arterioles and ramify into capillaries. (c) Blood flow after occlusions at different locations in the vascular hierarchy in downstream vessels.

Capillary perfusion assays. We demonstrate the ability to image single blood cell motion in cardiac capillaries enabling studies of heart blood flow at the resolution of the smallest blood vessels (Fig. 3). The animal received an intravenous injection of dextran-conjugated Texas-Red dye to label the vasculature. Red blood cells do not take up the dye so that they could be visualized as dark volumes within the fluorescent vessels (Fig. 3). One metric of microvascular function is the identification of stalled or plugged capillaries in image stacks (Fig. 3c). In brain, we find that chronic inflammation from Alzheimer's disease or alterations of hematocrit can cause leukocytes to plug individual capillaries. In brain occlusion of a capillary causes the RBC speed in the two downstream capillaries to drop to about 5-10% of the baseline value^{3, 35}. Even as far as four bifurcations away, the RBC speed is still less than half of baseline. The cumulative effect of multiple occlusions can be quite large. For example, based on the topology we estimate that if just 2% of capillaries in a region are blocked, the total tissue perfusion could drop by 20-30%. We observed monocytes plugging individual cardiac capillaries in our initial experiments (Fig. c).

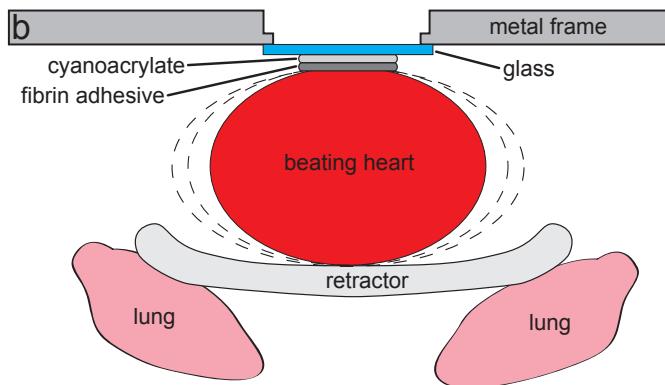
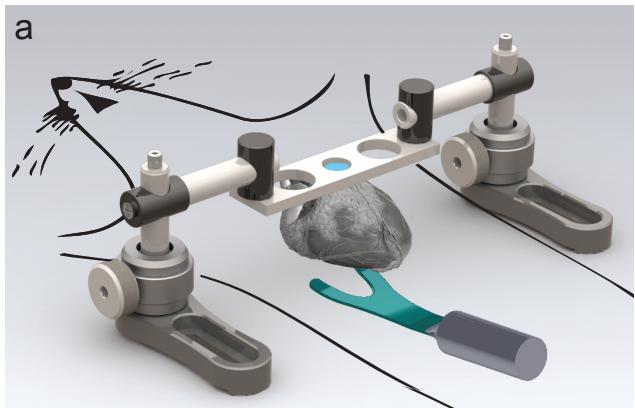


Figure 2 Mechanical stabilization of the heart *in vivo*. (a) Frame with glass window is positioned above heart in open-chest preparation. Retractor to isolate breathing is below. (b) Side view shows heart glued to glass window in a small area.

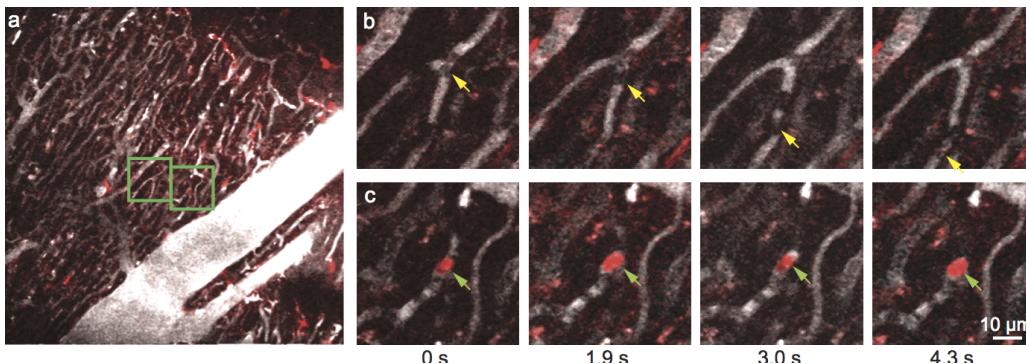


Figure 3. Capillaries in beating heart imaged with multiphoton microscopy. (a) Vasculature (white), monocytes (red). (b) Red blood cells (yellow arrow, black in vessel) can be seen moving through a capillary. (c) Monocyte plugging a capillary (green arrow).

Single vessel red blood cell motion varies with cardiac phase. By adjusting the delay between the R-wave of the ECG and the image acquisition time, we can take images at various times in the cardiac cycle. One limitation of multiphoton imaging is that because this relies on scanning the laser focus across the image area, the frame rate is limited. However, in addition to ECG-triggered image acquisition and optimizing scan parameters and image resolution for fastest frame rate with sufficient spatial resolution, we have established post-processing protocols to relate the imaging data to the cardiac phase. Using this technique, we use linescans⁴ to measure centerline RBC velocities in individual blood vessels as it varies with ECG (Fig. 4ab). Tissue flow (mL/min/tissue volume) can be determined by summing flow measured in individual vessels in a neighborhood. The topology of cardiac tissue is slightly different from cortex, so that we expect the exact values of flow changes after an occlusion to be different, but expect that the qualitative effect to be the same. Decreased flow downstream from occluded vessels could cause the gradual spread of compromised blood flow as the reduced flow causes inflammation that in turn causes capillary occlusions in the surround, further reducing blood flow

Single cell function across a cardiac cycle. Also using ECG-triggered acquisition and post processing, we demonstrate the measurement of action potentials in single cardiac myocytes of mice that express calcium-reporting fluorescent proteins (G-CaMP) (Fig. 4cd). In these mice we can also visualize the edges of each myocyte. With the triggered image acquisition, we can then measure the position of the myocyte edges and find the degree of contraction/thickening of each cell (Fig. 4ef). To our knowledge, this pilot data is the first time such a variety of measurements with single cell resolution can be made in the beating heart. The variability is high in this preliminary data, but we are showing a small number of measurements in order to demonstrate that we capture heterogeneity. Longer measurements per cell and continued

image processing improvements are expected to improve the resolution of these measurements. This enables us to study the cell-to-cell variability in cardiac function and quantify the relationship between decreases blood flow, action potential propagation, and contractility. This resolution will enable us to identify dysfunction based on heterogeneous cell failure, which is likely to be the case around small lesions.

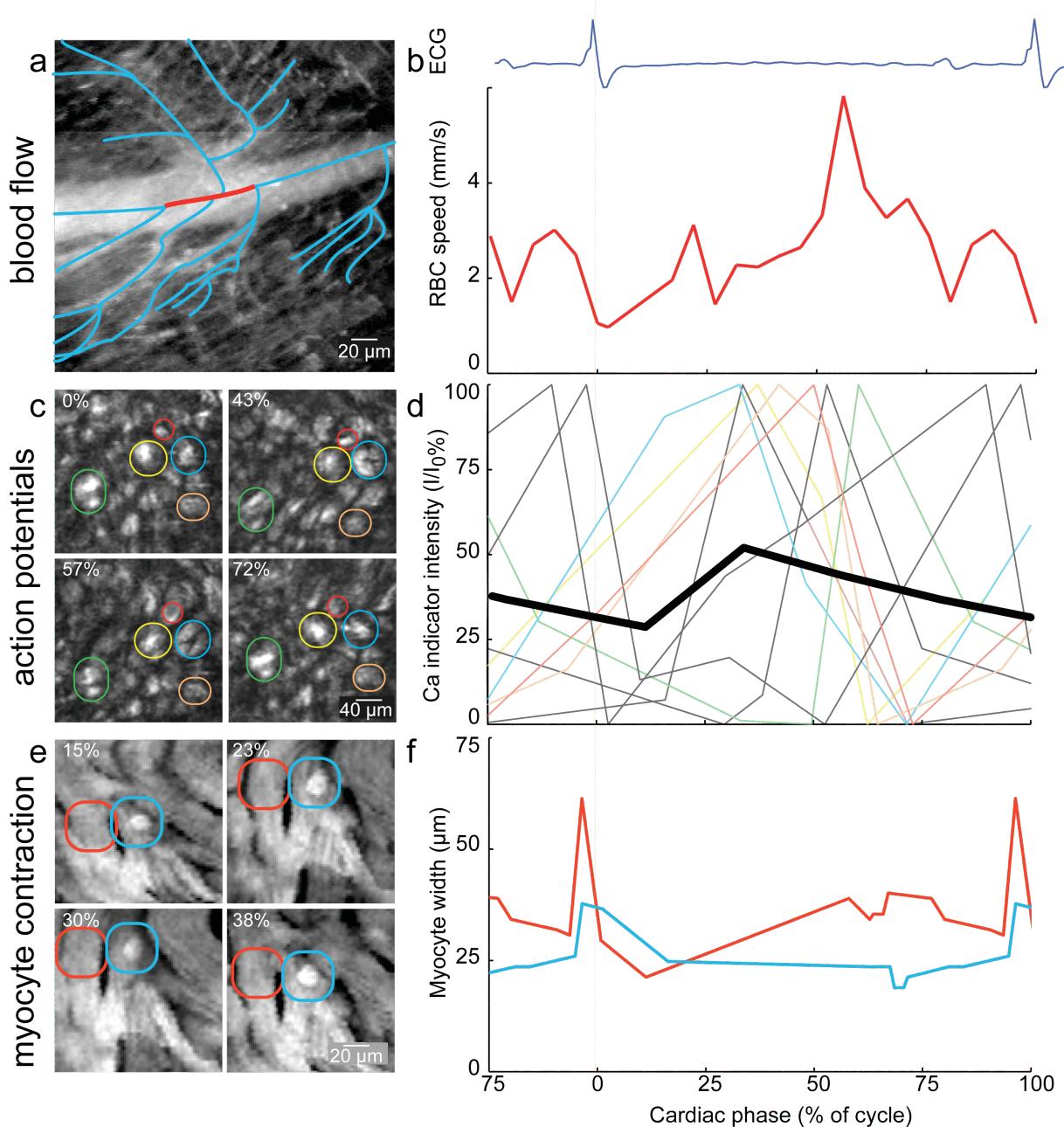


Figure 4. Functional measurements varying over cardiac cycle in the beating, in vivo heart . (a) Coronary arterioles and capillaries with tracings indicating connectivity. (b) Example blood flow measurement using line scan data from vessel traced in red in (a). Simultaneous ECG measurements were used to align signals to phase in cardiac cycle. (c) Action potentials are measured in mice expressing G-CaMP, a calcium sensitive fluorescent indicator, in myocytes. (d) Normalized fluorescence (proportional to $[Ca]$) in individual cells (fine lines) and averaged (thick lines). Colored outlines in (c) indicate location of cells in (d). (e) Myocyte shape change visualized in mice expressing GFP driven by a chick-actin promoter (CAG). (f) Width of cells outlined in (e) as a function of cardiac phase.

D. RESEARCH DESIGN AND METHODS

While several groups have made forays into using multiphoton microscopy in moving samples such as the beating heart^{36, 40}, our experience in quantitative imaging in brain enables us to perform measurements such as single capillary blood flow velocities and single cell calcium dynamics. In addition, we have developed new single-cell contractility assays. The mix of our experience with quantitative data acquisition and analysis and *in vivo* imaging enable us to make cellular measurements of interest to the problem of myocardial infarction.

Aim 1-Determine the type of vascular occlusion that causes myocardial microinfarction.

We test the hypothesis that occlusions of penetrating arterioles, defined as terminal vessels which branch off the surface coronary arteries and dive into the myocardial wall to the capillary beds, result in myocardial infarction. There are two types of penetrating arterioles⁴¹: class A are fine, branch quickly and feed the outer layers of the myocardial wall, while class B tend to be larger and run all the way to the endocardium. Based on our studies of blood flow changes after occlusions in brain, we expect that the lack of collateral connections between penetrating arterioles will result in blood flow deficits that can cause infarction and contractile dysfunction. With one exception, the vascular topology of the myocardium is similar to the cortex, so this expectation is well justified: Class B arterioles link to other B arterioles via connections that run parallel to the endocardium at the inside wall, a topology that is not found in brain⁴¹. The function of these connections is unclear because, unlike surface arterioles at the epicardium, these deep connections are predicted to be at low pressure and are therefore unlikely to be effective in acting as an alternate supply to the territory of an occluded penetrating arteriole.

Using multiphoton microscopy, we identify target arterioles as either class A or B penetrating arterioles. Vessels will be occluded using photodisruptive clotting with a high-power femtosecond laser³. Control experiments will have the same amount of imaging, but no irradiation with the high-power femtosecond laser.

Macroscopic contractile dysfunction: We test whether these lesions cause a gradually increasing amount of contractile dysfunction. We use ultrasound imaging for wall thickness after occlusions of both A and B penetrating arterioles and control experiments with no occlusions. Ultrasound imaging is used clinically, and an objective of this experiment is to help us relate a measurement that is clinically relevant (wall thickness) to multiphoton microscopy and histological findings. As another measurement of function, we will measure blood pressure via a femoral catheter. Time-lapsed ultrasound imaging and blood pressure measurements will continue for 3 hours to measure the time dependence of the lesion. We expect that we can make lesions that are small enough that the overall contraction of the heart at short time periods does not change much so that blood pressure measured in the periphery would be stable. Such a model would be ideal because we would not have to consider change in preload or afterload in understanding how contractility changes.

Infarction dynamics: To test whether these lesions cause infarction and if these lesions tend to grow over time as suggested by clinical observation, animals will be euthanized at 15-20 minutes, 1 hour and 3 hours after occlusion. Hearts will be extracted. We will use TTC and histological (H and E staining) methods to then measure infarction size. Immunohistology for specific inflammatory (LFA-1 and Mac-1)⁴⁰ and apoptotic markers (e.g. TUNEL and caspase 3) will be used to measure and compare the damage progression.

Interpretation - We expect infarction and contractile dynamics to track changes in perfusion. If this is the case then in the initial phase of MI, dysfunction is caused by lack of blood flow. In contrast, in chronic ischemia with "hibernating" tissue, the flow deficit is an effect rather than a cause. If our conjecture is right, then aggressively targeting the mechanisms for the propagation of vascular deficits (see Aim 2) could be prophylactically targeted in patients. In these experiments, our goal is to establish a model lesion for use in the following experiments that qualitatively resembles the dynamics thought to occur in patients. In addition, these data further our understanding of how different patterns of vascular connectivity and network structure confer vulnerability to occlusions.

Based on experiments in brain measuring blood flow changes⁵, we expect that about groups of 10 lesions would let us observe a difference between severe and moderate ischemia expected to be caused by occlusions of class B and A arterioles, respectively. This would require 30 animals for the contractile study. Some of these animals would be used for the final time point in the infarction study and we expect the infarction measurements to be less noisy than the contraction measure so that we would need 5 animals per vessel group (A,B, control), at three time points, totaling about 30-40 animals.

Potential pitfalls - We seek a myocardial infarction that emanates from a core to a surrounding area and in which these different regions are accessible to our imaging field. The ideal model would have a gradual progression of normal to stunned or hibernating myocardium within the timescale of an imaging experiment. We may not be able to optimize for all these aspects of the human disease in a single model. In this case, we could use several different types of injury to comparatively study different aspects of disease progression. If a single penetrating arteriole occlusion does not have a measurable effect we can use an alternate model of phot thrombosis by rose bengal. This model has the disadvantage that multiple vessels, including capillaries, will be occluded all at once so that this model maybe more ischemic than a spontaneously occurring embolus. In brain, these rose bengal lesions do demonstrate a region of compromised flow outside a necrotic core, so that we can still study the a perfusion-defined region-at-risk.

Aim 2-Identify the microvascular dynamics that contribute to lesion expansion over time. We will use Aim 1 to choose a single type of vessel for occlusion to achieve a consistent lesion model for the following experiments.

Vasoconstriction/capillary collapse: Using multiphoton imaging we measure the centerline RBC speed and the diameter of individual vessels ranging from large surface communicating arterioles and venules to capillaries before occlusion of a target vessel and after 20 minutes, 1 hour, 2 hours and 3 hours. The measured vessels will be subcategorized into vessels that are upstream or downstream of the vessel targeted for occlusion or not connected to the target vessel by making three-dimensional maps of the vascular architectures. Additionally, each measured vessel will be described by the geometrical distance (straight line) and also the topological distance (the number of vessel branches) to the target. At the end of the experiment, we apply a vasodilator, acetylcholine (10 mM)⁵, to confirm the involvement of active vessel vasoconstriction by reversing it with vasodilation.

These measurements enable us to confirm or find deviations from our working hypothetical sequence of events. If endothelial function is normal, we would expect that the myogenic reflex would cause vessels downstream from the occlusion to dilate (because they experience sudden loss of perfusion pressure). However, with some time under ischemia (likely minutes)

as necrosis and inflammation develop the endothelial cells will become dysfunctional or damaged and could lead to contractile signaling that leads to vasospasm in arterioles or precapillary arterioles.

In vessels without active contractile elements such as capillaries and venules, we hypothesize that the change in the difference between tissue and lumen pressure could drive lumen collapse. Myocyte swelling due to altered ionic balances could squeeze vessels, and we would expect to see diameter decreases in capillaries. Our methods allow us to resolve 0.5 μm changes in brain⁵. Although the motion of the heart would make this measurement a bit noisier, we expect to be able to detect similar changes based on the image quality in our preliminary data. In capillaries, the lumen does not have to completely collapse in order to significantly obstruct RBC motion. Because RBCs normally have to deform to squeeze through capillaries, a small change in lumen diameter or in lumen “bumpiness” can alter flow drastically. Based on brain experiments, we can measure about 15-25 vessels per experiment, so to get significant number we expect to need about 10 animals for each group (lesion, control). Potential pitfalls include the likelihood that ACh may not dilate the vessels, but in this case we can try papaverine and activating smooth muscle cells directly.

Leukocyte plugging - Because necrosis develops rapidly in the heart tissue, it is likely that cytokines and other signaling molecules will recruit leukocytes to the damage area within hours. We hypothesize that the neutrophil accumulation can lead to capillary plugging and venule adhesion. Others have noted venule adhesion⁴⁰, however capillary plugging has been harder to study due to the need for higher resolution and deeper imaging. In brain inflammation we have noted that capillary plugging can happen without much venular adhesion. While venule adhesion would increase vessel resistance due to a decrease in the available lumen for blood flow, the impact of a capillary plug results in a total stoppage of flow in that vessel. Our models of brain blood flow (albeit with a different network connectivity than heart) suggest that a 2% incidence of plugged capillaries could drop flow in a tissue by 20-30%.

In our studies of single vessel occlusions, we find that the flow in the immediate downstream capillaries is extremely slow, but still moving. This means that there is an opportunity for leukocytes to be attracted to the damaged tissue downstream from the occlusion. In addition, it appears from qualitative inspection that the capillary territories of individual terminal penetrating arterioles interdigitate and may also connect. This provides another route, with unrestricted blood flow, for leukocytes to access the damaged tissue and get captured by adhesion molecules expressed on endothelium.

It is likely that the number of leukocytes captured by inflamed capillaries is not as great in our permanent occlusion models as it would be after a reperfusion model that restores blood flow to the ischemic area^{42, 43}. However, our previous experience with mild inflammation in the brain suggests that even without reperfusion, leukocyte adhesion could affect blood flow and contribute to an expanding region of ischemia. The majority of leukocytes that adhere to myocardial venules within hours after insults such as transplantation or ischemia are neutrophils, although monocytes will also start to accumulate. To test for neutrophil adhesion we propose to deplete neutrophils with systemic administration of GR-1 antibody⁴⁴ one day before lesioning and imaging.

We use image stacks before and after lesions (Fig. 2) to quantify the total number of cardiac capillaries and the incidence of plugged capillaries. Leukocytes can be labeled *in vivo* with rhodamine-6G injections. Based on brain experiments, we can quantify leukocyte adhesion and stalling about 300 capillaries in 10 minutes, but we will need to compare across several

points in the ECG cycle (Fig. 3b). To get significant number we expect to need about 20 animals for each group (lesion, no lesion, lesion with GR-1 leukocyte depletion).

Interpretation – The relationships between ischemia, inflammation, altered contractility can drive a positive feedback loop that worsen myocardial infarction. The novel capabilities of our measurements enable us to look for the causes of progressively decreasing blood flow at the microvascular level, which has been very difficult to do previously.

Potential pitfalls – it might be that neutrophil depletion does make much of a difference. We can also try blocking other targets with antibodies that mediate receptor-ligand bonding between other types of leukocytes and endothelium (e.g. LFA-1, ICAM).

Aim 3 - Investigate the progression of excitation-contractility dysfunction.

Using the lesion model selected in Aim 1 which exhibits perfusion contraction matching (both decreased blood flow and contraction) we now use the superior spatial resolution of multiphoton microscopy to measure the microscopic dynamics underlying the functional deficit. *We measure the three-way coupling and uncoupling between perfusion, excitation and contraction due to penetrating arteriole occlusion.*

Single cell calcium imaging for AP detection with contractility measurements: Using G-CaMP animals, we characterize APs by imaging intracellular calcium dynamics. The change in fluorescence due to action potentials is small enough so that we can also use the fluorescence to measure the cell size throughout the cardiac cycle. Before and after penetrating arteriole occlusions (using vessel selection parameters optimized in Aim 1), we measure both calcium and contraction as a function of cardiac cycle (Fig. 4). By comparing the shape of the calcium transient and the contraction changes across the cardiac cycle in the same cell, we can look for both changes in each measurement and changes in the coupling. Our microscopy also gives us the unique capability to measure the changes as a function of depth in the tissue and we expect that lateral propagation of dysfunction will have different rates and patterns than vertical spread because the myofibril alignment is mostly horizontal.

Interpretation - We expect to find several features. First, although on average, the progression of dysfunction will likely spread outward from a small region in the subendocardium to the epicardium as well as laterally, we expect there to be great heterogeneity from cell to cell. Because the myocytes are coupled together by gap junctions as well as through mechanical coupling⁴⁵, we expect that changes in AP and contraction may spread along connected fibers. Altered APs and/or contractility in one cell could affect the neighboring cells. We expect to find that dysfunction depends on both the distance from the occluded vessel and time after occlusion, with severity and spatial extent increasing with time. AP alteration (likely a shortening of duration) will likely be most altered near the necrotic core of the lesion with contractility following a similar pattern. Controversy in the literature on the relative importance of AP change and calcium handling alterations¹⁴ suggests in addition to species difference, it could be that experimental techniques are not sufficient to adequately measure this. Using multiphoton microscopy, we will like find that there is a mix of these phenomena all in the same lesions, but heterogeneous from cell to cell.

Second, we expect to find that the expansion of the regions of dysfunction to correlate with regions of decreased blood flow or vasospasm from Aim 2. If this is confirmed, this would confirm that ischemia drives the conversion between hibernating and stunned myocardium and also may cause infarction spreading. If this is not confirmed, then other factors (perhaps

cytokines, ROS, protein degradation, etc.) that determine this transition would need to be elucidated in future studies.

Potential pitfalls - These experiments (contraction and APs) are somewhat noisier than blood flow measurements, so we expect to need about 20 animals per group (lesion and without lesion). This number could be lower if we find we can do simultaneous AP and contraction measurements. Potential pitfalls: If labeling in the G-CaMP mice is insufficient to measure contractility at the same time as calcium, we can use the vascular and also other transgenic mice such as the CAG mouse (Fig. 4d) as alternate methods of measuring contractility. Also if blood flow on the capillary level is extremely heterogeneous, we may have to do experiments in which we measure all three parameters (RBC speeds, calcium and contractility) in the same experiment rather than correlate the average trends of each. While this would slow down the experiment, there is no fundamental reason this cannot be done.

Future studies

This proposal would enable us to do an initial study of mechanisms of myocardial infarction. Because this is the first study using our newly piloted methods of imaging and quantifying single-cell cardiac dynamics, we chose to limit this proposal to permanent occlusion observed over acute time scales. Future studies could expand to chronic models in which the progression of damage and perhaps healing is monitored on the timescale of days to weeks. The addition of a reperfusion model would enable future studies of chronic hibernating and stunned myocardium. Importantly, the temporal and spatial resolution of our new tools enables us to ask questions about microscopic lesions and dynamics. Heterogeneity in tissue response can now be uncovered. Since this is in the context of the full, living animal, we will be able to study the interaction of cardiac function and health with systemic effects such as blood flow and inflammation. The combination of the whole organism with cell-resolved measurements of functions enables us to study complex phenomena that may have been masked in simplified or reductionist preparations such as extracted tissues and isolated cells.

E. ETHICAL ASPECTS OF THE PROPOSED RESEARCH

In our lab, we have made every effort to insure the health and safety of the researchers. All personnel will be appropriately trained by the Cornell Center for Animal Resources and Education (CARE) in the safe handling of rodents and the use of anesthesia and euthanasia drugs and other agents. This research necessarily involves the use of several high-power, Class IV, laser systems. All personnel will be trained personally by the P.I. in the safe alignment and use of these systems, and protective laser eyewear will be required in the laser laboratory at all times.

We have chosen rodents for our experimental study because they represent the simplest laboratory animal that has all the necessary features to study mammalian myocardial infarction. We make every effort to minimize the pain and discomfort of our research animals, and strive to get as much useful data from each animal as possible. For example, animals used for characterization of *in vivo* events following a small-scale vascular lesion will also be added to the pool of animals on which post-mortem histological studies are performed.