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Endogenous contrast blood flow imaging in embryonic hearts using hemoglobin contrast subtraction angiography

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Abstract

The genetic basis of congenital heart disease (CHD) is yet to be defined, and the interactions between the malformed heart and biomechanical cardiac performance remain poorly understood. Functional optical imaging enables detailed biomechanical phenotyping of cardiac dysfunction in small animal models, which in turn enables specific gene-phenotype relationship. We have developed a new microangiography technique based on flow imaging using endogenous hemoglobin contrast enabling *in vivo* assessment and biomechanical phenotyping of *Xenopus tropicalis* embryonic heart. We demonstrated that hemoglobin contrast angiography can be used to quantify physiological response to treatment with well-established cardioactive drugs.

Congenital heart defects are present in approximately 1% of live births and remain a significant cause of mortality.¹ Unfortunately, the etiology of congenital heart defects remains incompletely understood. The pairing of small animal models of congenital disease with functional heart imaging is shedding new light on abnormal heart development which, in turn, is advancing clinical treatments of congenital heart defects (e.g., fetal balloon dilation of aortic stenosis, materno-fetal hyperoxygenation for hypoplastic left heart syndrome).² Therefore, continued advances in the treatment of congenital heart disease rely on continued advances in functional imaging of small animal hearts.

One promising area of research enabled by functional optical imaging is the quantitative imaging of heart dysfunction in small animal models of congenital heart disease. Quantitative imaging involves deriving quantitative parameters (e.g., stroke volume, ejection fraction) from functional imaging of embryo hearts and establishing specific gene/phenotype and drug/phenotype relationships. Flow imaging can be used to extract these parameters, which require flow contrast mechanisms that can be classified as endogenous or exogenous. For example, fluoroscopy-based clinical cardiac angiography uses exogenous radio-opaque dyes to generate contrast between blood and surrounding tissue. For small animal optical

imaging, cardiac angiography typically requires injection of optically dense or fluorescent dyes. However, microinjection is technically challenging, which limits the use of angiography in optically-accessible small animal models of congenital heart disease such as *Xenopus*, zebrafish and chick embryo.

It is worth noting, though, that hemoglobin itself is a potential source of endogenous absorptive contrast given that hemoglobin has an absorption spectrum that varies over several orders of magnitude over visible wavelengths (Fig. 1). In this study we demonstrate hemoglobin contrast subtraction angiography (HCSA), a novel optical angiography method that exploits the wavelength-sensitive absorption of hemoglobin as a source of endogenous flow contrast. HCSA simultaneously enhances blood while suppressing background features and empty heart chambers. It can also encode more than one absorptive species in the directionality (i.e., sign) of contrast enhancement. We demonstrate the technique in *Xenopus tropicalis* embryos, an experimentally tractable and genetically manipulable animal model of congenital disease.³ We also demonstrate that several different quantitative phenotypes can be extracted from HCSA movies in the setting of pharmacologic perturbation.

Subtraction angiography is a general image processing technique for suppressing background features while highlighting flow features generated by a specific contrast mechanism.⁴ Subtraction angiography requires a background or mask image I_{mask} . For traditional x-ray based angiography, the mask image is obtained prior to injection of exogenous contrast. For a time series of N x-ray images $I[n]$, $n \in \{1 \dots N\}$, with contrast injection at $n=2$, $I_{\text{mask}}=I[1]$. As such, I_{mask} may not be correctly registered with subsequent images $I[n]$. Color cameras employing Bayer filters⁵ acquire three images (one for each color channel) that are co-registered in space and time, suggesting that one of the three images can be used as the background image. In the case of hemoglobin, which has weak absorption at red wavelengths compared to the rest of the visible spectrum (Fig. 1), the red channel image can serve as I_{mask} , assuming that the tissue absorption spectrum is relatively uniform across the visible spectrum assuming that the blood-free heart has similar spectral properties with respect to the background tissue and that blood is similar to tissue in the red channel. For a time series of RGB color images $I_c[n]$, C , $I_R[n]$ can serve as a mask image $I_{\text{mask}}[n]$ for each acquisition. Thus, the subtraction angiogram time series is given by $I_{\text{HCSA}}[n] = I_G[n] - kI_R[n]$, where k is a normalization constant that accounts for spectral variations in source intensity and in detector responsivity. k was calculated as the ratio of mean pixel values in a background region of interest.

In this study, a color camera was c-mounted to a stereomicroscope that used white light sample illumination. A Canon EOS 5D Mark II DSLR was used to image the hemoglobin solution in Fig. 1 and an IDT/Redlake N4 was used for high-speed (200 or 500 frames per second) embryo heart imaging. *X. tropicalis* embryos were generated according to established protocols. Stage 45/46 *Xenopus tropicalis* embryos obtained from Khokha lab, immobilized in low-melt agarose and imaged at 20 C.

A hemoglobin phantom was made filling a wedge-shaped gap between two glass slides with 5g/dL hemoglobin A₀ solution (Hemoglobin A₀, Ferrous Stabilized [Sigma-Aldrich] dissolved in phosphate buffered saline). As expected, the thicker parts of the wedge yield stronger HCSA enhancement attributable to higher total absorption by hemoglobin (Fig. 1A). Similar enhancement is seen in an embryonic heart (Fig. 1B-Media 1). For both cases hemoglobin is qualitatively more visible in the blue and green channel images than it is in the red channel image. Both observations are consistent with the hemoglobin absorption spectrum, which is weakest in the ~600-700 nm range. Thus, when hemoglobin is in the ventricle, the green (or blue) image can serve as a contrast-enhanced image for subtraction angiography and the red image can serve as the spatially and temporally-registered mask

image. HCSA produces a grayscale image in which endogenous hemoglobin is strongly enhanced compared to the background. The HCSA image shows pumping of blood from the ventricle to the conus arteriosus thereby enabling dynamic flow imaging of hemoglobin and characterization of flow *in vivo*.

The principle of HCSA can be generalized to perform endogenous contrast imaging of more than one absorptive species. Specifically, the direction (i.e. sign) of the contrast enhancement in one color channel compared to another can be used for multiplexing the detection of different absorptive species in one image. The gallbladder is located under the right inferior pole of the ventricle and is filled with green bile (Fig. 2a). HCSA highlights the opposite enhancement of green bile when compared to red hemoglobin (Fig. 2b and c). Pixel intensity values from the individual RGB color channels do not show contrast among bile, blood, and background tissue. However, HCSA shows clear contrast enhancement of blood and bile. Also note that the empty heart generates a similar HCSA signal compared to background tissue.

One advantage of contrast imaging is the ability to quantify the number of enhanced pixels and then relate that number to important anatomic and physiologic parameters. Importantly, counting the number of enhanced pixels allows for the measurement of areas and volumes of structures with complex shapes. Embryo hearts are often assumed to be a prolate spheroid when estimating chamber volumes. However, such an assumption is clearly violated when the heart is trabeculated, which imparts an irregular shape to the ventricular lumen since blood can be present between thin trabeculations (orange arrow in Fig. 2).

To demonstrate the utility of HCSA, we quantified several different quantitative phenotypes in a *Xenopus tropicalis* embryonic heart before and after treatment with a cardioactive drug (Fig. 3). We acquired high-speed (200 frames per second) color movies of a *Xenopus tropicalis* heart before and after treatment with verapamil, an L-type calcium channel blocker. Since the embryo was immobilized in low-melt agarose, and since drug treatment was administered without removing the embryo from the microscope stage, embryo position and lighting conditions were essentially the same before and after drug treatment.

Ventricular hemoglobin blush was defined as the number of hemoglobin-enhanced pixels in the ventricle. A pixel was identified as hemoglobin-enhanced based on a threshold value in an HCSA image. The threshold was constant across all images within a movie and between the before and after movies. The ventricle was identified using a manual region of interest, and hemoglobin blush was plotted as a function of time (Fig. 3). Using hemoglobin blush curves, several quantitative phenotypes were estimated, including end diastolic area (EDA), end systolic area (ESA), stroke area (SA), ejection fraction (EF), and systolic and diastolic inflow rates.

Prior to treatment the ventricle appears to completely empty during systolic contraction (Fig. 3, left panel). After treatment the ventricle appears to fill with more blood during diastole and also appears to have less efficient systolic pumping (Fig. 3, right panel). These subjective assessments are verified in quantitative analysis of the HCSA images. The curves reach a maximum during diastolic filling and reach a minimum during systolic pumping. Prior to verapamil treatment, the end systolic area (ESA) is near zero, yielding an ejection fraction (EF) of ~100% ($EF = SA/EDA$; EDA, end diastolic area; $SA = EDA - ESA$). After verapamil treatment, the ESA is nonzero, the EDA significantly increases, and the EF is about 50%.

Given the high frame rates of the color camera, we additionally were able to estimate two-dimensional rates of diastolic filling and systolic pumping. These rates can be estimated

from the slope of the upswing (diastolic filling) and downswing (systolic pumping) of the hemoglobin bluish curve. Before (after) treatment with verapamil the diastolic inflow and systolic inflow rates were $145 \mu\text{m}^2/\text{ms}$ ($110 \mu\text{m}^2/\text{ms}$) and $175 \mu\text{m}^2/\text{ms}$ ($90 \mu\text{m}^2/\text{ms}$), respectively. Thus, HCSA is able to quantify dynamic dysfunction in myocardial contraction/pumping and relaxation/filling.

Our HCSA method does share similarities with other methods to enhance certain color features of images (multispectral imaging⁶, narrow-band imaging⁷). For example, narrow-band endoscopic imaging generates color images using sample illumination at two or three narrow wavelength bands to highlight vasculature against surrounding tissue. Although the narrow-band approach increases the visibility of vessels in color images, it ultimately does not generate background-free vascular images or quantification of blood content in a region of interest. HCSA provides grayscale movies amenable to qualitative analysis as well as quantitative binary (i.e. one-bit) images that serve as maps of hemoglobin. On the other hand, because multispectral imaging techniques use detectors with much finer spectral resolution than HCSA does, multispectral imaging can obtain spectroscopic information (e.g. percent hemoglobin oxygen saturation) that is inaccessible to HCSA.

Overall, HCSA is a novel endogenous contrast imaging method that enables quantitative, *in vivo* measurements of abnormal embryo heart physiology. From an experimental biology perspective, hemoglobin contrast subtraction angiography is complementary to crosssectional methods such as optical coherence tomography/microscopy and confocal microscopy. Clinical cardiology has demonstrated that having complementary imaging modalities (e.g., echocardiography and cardiac angiography) is essential since each modality yields different kinds of physiological information relevant to complex disease states. Furthermore, since noncompaction ventricular cardiomyopathy is characterized by abnormal ventricular trabeculation⁸, and since HCSA can visualize intra-trabecular blood, it is a potential modality for studying this disease in animal models. Future work will focus on using HCSA to establish specific gene-phenotype relationships in the genetically-modified *Xenopus tropicalis* heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Overview of HCSA. First row (A) shows light absorption spectrum of Hb. Second row (B) shows wedge-shaped hemoglobin phantom. Third row/Media 1 (C) shows an embryonic heart of *Xenopus* in mid-systole, which is midway through ejecting blood out of the ventricle. The columns contain, in order, original image, blue, green and red channel, and HCSA image generated by subtracting red from green channel. c, conus arteriosus; v, ventricle.

Fig. 2.

(a) Stage 46 tadpole heart and gallbladder shown from antero-posterior view at the end of diastole (b) Corresponding grayscale HCSA (c) False-color HCSA image with black representing zero contrast, enhancement highlights the opposite enhancement of green bile when compared to red hemoglobin. (d) Grayscale HCSA image of the same heart at the end of ventricular systole with essentially no blood in the ventricle. Spiral valve situated within the conus creating differential flow to systemic and respiratory circulation. (e) HCSA pixel intensity values of gallbladder, conus arteriosus and background plotted along the line of interest in Fig. 2d during systole and diastole. b, background; c, conus arteriosus; gb, gallbladder; v, ventricle; vv, vein; orange arrow indicates blood between thin trabeculations; sv, spiral valve.

Fig. 3. Quantitative physiological response to embryo heart drug treatment assessed using HCSA. (Media 2) ESA, end systolic area; SA, systolic area; EDA, end diastolic area; EF, ejection fraction.