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In Situ Multi-Photon Fluorescence Microscopy for Functional Screening of Intracardiac Cell Implants

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The intrinsic renewal rate of the adult mammalian myocardium is insufficient to compensate for myocyte loss during cardiac disease. Transplantation of donor myocytes or myogenic stem cells has been shown to improve cardiac function, but it has not been clear whether this effect results from direct contribution of functionally integrated donor cells. Active contribution of cellular grafts to the overall contractile activity minimally requires the ability of individual donor cells to develop intracellular calcium ($[Ca^{2+}]_i$) transients in phase with the undamaged host myocardium. We have previously developed a multi-photon laser scanning microscopy (MPLSM)-based $[Ca^{2+}]_i$ imaging assay that allows us to quantitate, at the level of individual cells *in situ*, the degree of functional coupling between transplanted donor cell types and resident host cardiomyocytes.

In an initial proof-of-concept study, we transplanted single cell suspensions of embryonic day 15 ventricular cardiomyocytes from transgenic mice expressing enhanced green fluorescent protein (EGFP) under control of the cardiomyocyte-specific α-myosin heavy chain promoter (MHC-EGFP mice) into the left ventricular wall of non-transgenic adult mice. Hearts were harvested 1-5 weeks post engraftment, perfused in Langendorff-mode, loaded with the Ca^{2+}-sensitive fluorophore rhod-2, and subjected to MPLSM imaging for assessment of action potential-evoked $[Ca^{2+}]_i$ transients in EGFP-expressing donor and non-expressing host myocytes simultaneously. Cytochalasin D (50 μmol/l) was used to uncouple contraction from excitation. A representative example of MPLSM images taken from a heart 5 weeks after cellular transplantation is shown in Figure 1. The frame-mode image (Figure 1A) was obtained during continuous electrical stimulation at a remote site from the field of view. Periodic increases in rhod-2 fluorescence, reflecting electrically triggered $[Ca^{2+}]_i$ transients, occur simultaneously in donor and host cardiomyocytes (which appear yellow due to the overlap of green EGFP and red rhod-2 fluorescence). The line-scan image (Figure 1B) produced by vertically stacking successive line scans along the white line in panel A as well as the time plot of spatially averaged rhod-2 fluorescence changes (Figure 1C) demonstrate 1:1 association of rhod-2 transients in host cardiomyocytes (cells 1, 4 and 7) and in donor cardiomyocytes (cells 2, 3, 5 and 6) during spontaneous sinus rhythm as well as during stimulation at 2 and 4 Hz, suggesting homo-cellular (donor-donor) and hetero-cellular (donor-host) coupling. Donor and host myocyte $[Ca^{2+}]_i$ transient kinetics were indistinguishable from each other (Figure 1D). All 177 imaged donor myocytes (from 7 individual animals) that were in physical contact with host myocytes were found to functionally couple. Overall, these experiments support the utility of the MPLSM-based assay for quantitative assessment of donor cell integration and further, the ability of fetal cardiomyocytes to perfectly integrate with the recipient myocardium.

We subsequently tested other donor cell types for their ability to functionally couple with host myocytes following intracardiac delivery. Primary skeletal myoblasts expressing EGFP were transplanted into

uninjured mouse hearts. MPLSM $[Ca^{2+}]_i$ imaging of explanted hearts 2 to 6 weeks later revealed the presence of action potential-induced $[Ca^{2+}]_i$ transients in EGFP-negative host myocytes, whereas no $[Ca^{2+}]_i$ transients were detectable in neighboring donor-derived myocytes. Electrical field stimulation of the intact heart restored $[Ca^{2+}]_i$ transients in the latter, indicating that lack of donor-to-host cell electrical communication rather than malfunction of the excitation-induced Ca^{2+} release mechanism underlies the absence of $[Ca^{2+}]_i$ cycles in the skeletal muscle graft. Immunohistological analyses demonstrated lack of connexin43 (Cx43) expression at the cardiac-skeletal muscle junction, corroborating the functional analyses. To examine whether ectopic Cx43 expression in skeletal muscle grafts enables their electrical coupling following intracardiac delivery, we injected EGFP-expressing C2C12 myoblasts carrying a transgene composed of sequences encoding the muscle creatine kinase (MCK) promoter followed by Cx43 cDNA. Intense anti-Cx43 immunoreactivity was detected throughout MCK-Cx43 muscle grafts at 2 to 6 weeks after transplantation. However, $[Ca^{2+}]_i$ transients in response to propagated action potentials were restricted to donor-derived myocytes located alongside the graft-host border, suggesting that Cx43 expression did not implement uniform electrical integration of myotube grafts.

Adult bone marrow cells have previously been hypothesized to give rise to functional *de novo* myocardium after their engraftment into the infarct border zone. Accordingly, we transplanted low-density mononuclear cells, c-kit-enriched cells, or highly enriched hematopoetic stem cells prepared from adult transgenic mice ubiquitously expressing EGFP, into the peri-infarct zone of non-transgenic mouse hearts ~5 h after coronary artery ligation. MPLSM of explanted hearts 9 days later failed to detect $[Ca^{2+}]_i$ transients in any of >3,000 donor cells screened along the infarct border zone, whereas periodic $[Ca^{2+}]_i$ transients were readily visible in surviving border zone host myocytes, suggesting that adult bone marrow-derived cells have only limited capacity to transdifferentiate into cardiomyocytes.

Our more recent studies employed MPLSM $[Ca^{2+}]_i$ imaging to interrogate the functional fate of human embryonic stem cell (hESC)-derived cardiomyocytes cells following their intracardiac injection. EGFP-expressing hESC-derived cardiomyocytes were obtained by directed differentiation and engrafted into the heart of adult mice. Hearts were harvested between 18 and 97 days post-engraftment and imaged as described above. The hESC-derived cardiomyocytes were typically observed to be closely juxtaposed with host cardiomyocytes (Figure 2A). When imaged in line-scan mode (Figure 2B), transient increases in rhod-2 fluorescence were detected in host myocytes (cells H1 and H2), as well as in hESC-derived myocytes (cells D1 and D2); however, donor and host transients were found to be out of phase (Figure 2C), indicating that the donor cells did not form a functional syncytium with the host myocardium.

In conclusion, MPSLM $[Ca^{2+}]_i$ imaging is a useful tool to define quantitatively the propensity of a variety of donor cells to couple stably and functionally with the recipient myocardium.

Figure 1.

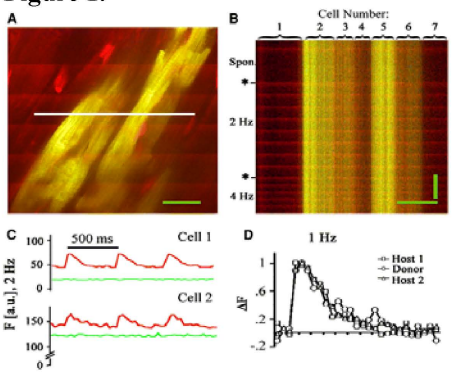

Figure 2.

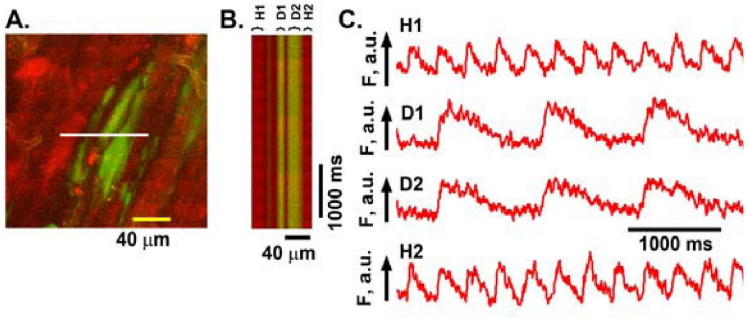

Figure 1 and 2: For explanation see text.

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