Published in final edited form as:

Pediatr Cardiol. 2012 August ; 33(6): 929–937. doi:10.1007/s00246-012-0314-8.

Functional screening of intracardiac cell transplants using two-photon fluorescence microscopy

Wen Tao¹, Mark H. Soonpaa^{1,2}, Loren J. Field^{1,2}, Peng-Sheng Chen², Anthony B. Firulli¹, Weinian Shou¹, and Michael Rubart¹

¹Riley Heart Research Center, Department of Pediatrics, Indiana University School of Medicine

²Krannert Institute of Cardiology, Division of Cardiology, Department of Medicine, Indiana University School of Medicine

Abstract

Although the adult mammalian myocardium exhibits a limited ability to undergo regenerative growth, its intrinsic renewal rate is insufficient to compensate for myocyte loss during cardiac disease. Transplantation of donor cardiomyocytes or cardiomyogenic stem cells is considered a promising strategy to reconstitute cardiac mass, provided the engrafted cells functionally integrate with host myocardium and actively contribute to its contractile force. We have previously developed a two-photon fluorescence microscopy-based assay that allows *in situ* screening of donor cell function following their intracardiac delivery. Here we review the techniques and summarize its application for quantitation of the extent to which a variety of donor cell types stably and functionally couple with the recipient myocardium.

Keywords

Cellular transplantation; myocardial regeneration; intracellular calcium regulation; two-photon fluorescence microscopy

Introduction

Although the adult mammalian myocardium exhibits a limited capacity to undergo regenerative growth [1,2], its intrinsic renewal rate is insufficient to compensate for myocyte loss during cardiac disease [3]. Transplantation of donor cardiomyocytes or cardiomyogenic stem cells is considered a promising strategy to restore myocardial mass [4,5]. Cellular transplantation-based approaches have been shown to improve cardiac function [5], however, it has not been clear if this effect reflects direct contribution of functionally integrated donor cells. Although indirect effects can clearly be of therapeutic value, reconstitution of lost contractile function remains the ultimate goal of cellular transplantation strategies. Active contribution of cellular grafts to the overall contractile force of the recipient heart requires the ability of individual donor cells to form a functional syncytium with the undamaged host myocardium. Accordingly, we have developed a two-photon laser scanning microscopy (TPLSM)-based imaging assay that allowed us to quantitatively assess, at the level of the individual cell *in situ*, the extent of functional coupling between transplanted donor cells and resident host cardiomyocytes. Here, we briefly review technical aspects of the imaging assay and summarize its use to interrogate the functional fate of different donor cell types following their direct intracardiac delivery.

Requirements for an *in situ* assay to screen functional integration of transplanted donor cells

Because transplanted donor cells can be located tens to hundreds of microns below the epicardial surface, one major requirement of the assay was its ability to assess functional aspects of donor cells at the cellular level deep within the myocardial wall. Although single-photon confocal microscopy readily provides the capability to generate thin optical sections within living biological specimens, two-photon excitation fluorescence imaging has the significant advantage of improved imaging depth relative to confocal microscopy, with virtually no loss in spatial resolution [6]. During two-photon illumination using rapidly pulsed, high-intensity, long wavelength light (>700 nm), the fluorophore excitation, and thus emission, is confined to an ellipsoid volume around the focal point of the objective lens, giving two-photon imaging its intrinsic three-dimensional resolution. The magnitude of the two-photon excitation volume critically depends on the numerical aperture of the objective lens. For example, using a 1.2 numerical aperture objective and an excitation wavelength of 850 nm, fluorophore excitation, and thus emission, is confined to less than femtoliter volumes around the focal point with less than 1 micron extension in the axial direction [7]. However, spherical aberration, resulting from a refractive index mismatch between the objective immersion medium and the sample, and light scattering due to refractive index heterogeneity within the sample tissue itself can give rise to a significant decline in spatial resolution of multiphoton fluorescence microscopy with increasing depth [8,9]. Adapting the refractive index of the immersion medium to the mean refractive index of the tissue [9] and/or using longer wavelength excitation light (to reduce light scattering) may improve spatial, specifically axial, resolution. Also, deconvolution algorithms employing depth-specific point spread functions have been suggested to attenuate the adverse effects of spherical aberration and light scattering on resolution [10].

The second major requirement of the assay was its ability to provide a quantitative read out of the degree of functional donor cell integration. In cardiomyocytes, transient increases in cytoplasmic free calcium concentration serve an indispensable role in linking electrical excitation and mechanical contraction. Consequently, if engrafted donor cells were to actively contribute to the overall contraction of the recipient myocardium, then their excitation-contraction cycles would be expected to occur in phase with those in the surrounding host myocardium. Thus, our assay employed entrainment of intracellular calcium ($[Ca^{2+}]_i$) transients in donor cells by electrically evoked $[Ca^{2+}]_i$ cycles in the surrounding host myocardium as a criterion for functional coupling.

A third major requirement of the assay was the ability to unambiguously distinguish donor and host cells in the living heart *in situ*. Towards this end, our transplant studies have employed donor cells that were genetically modified to express enhanced green fluorescent protein, EGFP.

TPLSM $[Ca^{2+}]_i$ imaging in Langendorff-perfused mouse heart

We first examined the capability of TPLSM to monitor action potential-evoked $[Ca^{2+}]_i$ transients at the single cardiomyocyte level in intact, Langendorff-perfused mouse heart using the calcium-sensitive fluorescent indicator rhod-2 [11]. Because motion artifacts due to cardiac contraction distort fluorescence signals and thus prevent accurate fluorescence-based measurements of dynamic changes in intracellular calcium in intact hearts, cytochalasin D (50 μ M) was continuously applied during image acquisition to uncouple contraction from excitation [12]. A representative example of electrically evoked $[Ca^{2+}]_i$ transients in an immobilized, isolated perfused mouse heart is shown in Figure 1. An electrical stimulus delivered at a site remote from the field of view results in an increase in

rhod-2 fluorescence intensity, corresponding to an action potential-related increase in cytosolic free calcium. The rhod-2 fluorescence rises simultaneously over the length of the scan line, indicating that the sarcoplasmic reticulum Ca^{2+} release activated by the propagating membrane depolarization is highly synchronized within as well as among individual cardiomyocytes. To resolve the time course of changes in $[\text{Ca}^{2+}]_i$, rhod-2-transients were also recorded in line-scan mode during continuous electrical point stimulation as shown in Figure 1B. The red line in panel A was repeatedly scanned at a rate of 32 Hz and the line-scans were stacked vertically to obtain the composite line-scan image. From these line-scan images, fractional changes in spatially averaged rhod-2 fluorescence intensity as a function of time were obtained for each cardiomyocyte along the scan line, as illustrated in Figure 1C. Normalization of action potential-evoked $[\text{Ca}^{2+}]_i$ transients reveal similar upstroke and decay kinetics in all three neighboring cardiomyocytes (Figure 1D), suggesting intercellular coordination of Ca^{2+} release and removal in the intact heart. Notably, high-resolution line-scan images of action potential-evoked $[\text{Ca}^{2+}]_i$ transients obtained from individual ventricular cardiomyocytes *in situ* in the presence of cytochalasin D revealed spatially uniform increases in dye fluorescence that were very similar to those observed in isolated ventricular cardiomyocytes in the absence of the excitation-contraction uncoupler, but under otherwise identical experimental conditions (stimulation rate, temperature, and extracellular calcium concentration; Figure 2), suggesting preservation of spatial $[\text{Ca}^{2+}]_i$ transient properties under the experimental conditions of our studies. Overall, these observations demonstrated the utility of TPLSM in conjunction with rhod-2 to monitor changes in cytosolic calcium concentration on a subcellular scale within buffer-perfused, electromechanically dissociated mouse hearts.

Physiological coupling of transplanted fetal cardiomyocytes to the host myocardium – proof of concept

Next, we examined whether donor myocytes were able to functionally couple with the host myocardium following intracardiac delivery [13]. Single-cell suspensions of embryonic day 15 ventricular cardiomyocytes from transgenic mice with cardiomyocyte-restricted expression of EGFP were injected into the left ventricular wall of non-transgenic adult mice. Hearts were harvested 1 to 5 weeks after engraftment and subjected to TPLSM imaging for assessment of action potential-evoked $[\text{Ca}^{2+}]_i$ transients in EGFP-expressing donor cardiomyocytes and non-expressing host myocytes simultaneously. A representative example of TPLSM images taken from a heart 5 weeks after cellular transplantation is shown in Figure 3. The full-frame mode image in panel A was obtained during continuous 2 Hz stimulation at a site remote from the field of view. Donor cardiomyocytes (which appear yellow due to the overlay of green EGFP and red rhod-2 fluorescence) are well aligned with and morphologically indistinguishable from EGFP-negative host cardiomyocytes. Cyclic variations in rhod-2 fluorescence, due to electrically evoked $[\text{Ca}^{2+}]_i$ transients, appear simultaneously in all cells along the scan line (including those with EGFP fluorescence). The line-scan image (panel B) produced by stacking successive line scans along the white line in panel A demonstrates that spontaneous rhod-2 fluorescence transients occurred simultaneously in EGFP expressing donor cardiomyocytes (cells 2, 3, 5 and 6) and host cardiomyocytes (cells 1, 4 and 7). This 1:1 association of rhod-2 transients in host and donor cardiomyocytes was maintained during remote point stimulation at either 2 or 4 Hz., as well as after the resumption of spontaneous sinus rhythm, suggesting homocellular (donor-to-donor) and heterocellular (donor-to-host) coupling. Time plots of spatially averaged traces for the red and green fluorescence present in cells 1 and 2 were generated from the line scan data (Fig. 3C). Only the donor cell exhibited EGFP fluorescence. There was no change in EGFP fluorescence in the donor cardiomyocytes during spontaneous and evoked depolarizations (confirming that cytochalasin D sufficiently eliminated motion artifacts).

The frequency dependence of the kinetics of $[Ca^{2+}]_i$ decline in donor cardiomyocytes was indistinguishable from that in neighboring host cardiomyocytes (panel D) indicating that removal of calcium ions from the cytosol was highly synchronized among host and donor cardiomyocytes. Furthermore, $[Ca^{2+}]_i$ transient kinetics in host cardiomyocytes located along intracardiac grafts did not differ from those of cardiomyocytes remote from the graft border, indicating that the presence of functionally coupled donor myocyte grafts did not appear to adversely affect host myocardium Ca^{2+} handling. The lack of delay in the onset of donor and host myocyte $[Ca^{2+}]_i$ transients, the similarity in their decay kinetics, and immunofluorescence evidence of connexin43 gap junction formation at the donor-host interface support the notion that both cell types are electrically coupled. Importantly, all engrafted donor myocytes that were also in direct physical contact with host cardiomyocytes exhibited $[Ca^{2+}]_i$ transient entrainment by the $[Ca^{2+}]_i$ cycles of juxtaposed host cardiomyocytes. Collectively, these observations supported the utility of the TPLSM-based assay to assess donor cell viability and function, and they constituted the first direct proof that donor cardiomyocytes are capable of forming a functional syncytium with the uninjured host myocardium following intracardiac transplantation. Furthermore, donor-derived cardiomyocytes possess functional attributes that are necessary to develop force in coordination with their immediate host neighbors.

Skeletal muscle grafts create spatial heterogeneity of Ca^{2+} handling

Transplantation of skeletal myoblasts into normal or injured hearts results in the formation of nascent myotubes. This process has been shown to ameliorate adverse postinfarction remodeling, resulting in retention of improved global cardiac function. Although both functional and structural studies had suggested that *de novo* myotubes remain electrically isolated from the recipient myocardium, the occurrence of life-threatening arrhythmias in patients prompted us to study myotube:host myocardium functional interactions in more detail using TPLSM [14]. Primary myoblasts expressing EGFP were transplanted into uninjured mouse hearts. Two to 6 weeks later, hearts were explanted and subjected to TPLSM imaging as outlined. A representative result is illustrated in Figure 3. Periodic increases in rhod-2 fluorescence intensity, reflecting action potential-induced $[Ca^{2+}]_i$ transients, are visible as ripple-like wavefronts in host cardiomyocytes, whereas no $[Ca^{2+}]_i$ transients are detectable in neighboring donor-derived myocytes. Electrical field stimulation of the intact heart readily evoked $[Ca^{2+}]_i$ transients in both host and donor-derived myocytes, indicating that lack of donor-to-host cell electrical communication rather than malfunction of the depolarization-induced Ca^{2+} release mechanism underlies the absence of $[Ca^{2+}]_i$ cycles in the skeletal muscle graft. A small fraction of EGFP-expressing, i.e. donor-derived, myocytes that were located exclusively along the graft-host junction exhibited $[Ca^{2+}]_i$ transients in phase with their neighboring host cardiomyocytes. $[Ca^{2+}]_i$ transient kinetics in these functionally coupled, donor-derived cells could markedly differ from those of their neighboring host cardiomyocytes, generating spatial micro-heterogeneity of $[Ca^{2+}]_i$ handling along the graft-host junction. These functionally coupled donor cells were observed to develop tetanic $[Ca^{2+}]_i$ elevations in response to incremental increases in the rate of stimulation, compatible with the notion that they retain phenotypical attributes of skeletal myotubes. Additional studies in independent hearts demonstrated that the prevalence and anatomical location of functionally coupled donor-derived myocytes matched those of donor-host cell fusion events. Overall, these data suggest that engraftment of skeletal myoblasts generated spatial heterogeneity of $[Ca^{2+}]_i$ signaling at the myocardial/skeletal muscle interface, most likely resulting, at least in part, as a consequence of spontaneous fusion events between donor myoblasts and host cardiomyocytes. Because cell-to-cell gradients in $[Ca^{2+}]_i$ have been implicated in the genesis of arrhythmogenic Ca^{2+} waves, experiments are currently underway to directly address the propensity of skeletal muscle graft-bearing hearts to inducible ventricular arrhythmias. Furthermore, a recent study

reported protection from inducible ventricular tachyarrhythmias in infarcted mouse hearts harboring connexin43-expressing skeletal muscle grafts [15]. Accordingly, experiments are underway to quantitate, at a cellular level *in situ*, the degree to which ectopic connexin43 expression in intracardiac skeletal muscle grafts enables stable electrical donor host connectivity and influence electrical and $[Ca^{2+}]_i$ signaling in the host myocardium.

Transplantation of myogenic stem cells

Adult bone marrow cells have previously been hypothesized to give rise to functional *de novo* myocardium following their engraftment into the infarct border zone in mice [16], sparking quick translation of this concept into clinical applications [17,18]. We investigated the ability of transplanted bone marrow cells to express attributes of functional cardiomyocytes *in situ*, using TPLSM [19]. Specifically, we determined whether bone marrow-derived cellular grafts could develop $[Ca^{2+}]_i$ transients in phase with the host myocardium following direct injection into ischemically injured heart muscle. Low-density mononuclear cells, c-kit-enriched cells, or highly enriched hematopoietic stem cells were prepared from the bone marrow of adult transgenic donor mice ubiquitously expressing EGFP. The cells were engrafted into the peri-infarct zone of non-transgenic hearts ~5 hours after ligation of the left anterior descending coronary artery. Hearts were harvested 9 days later and subjected to TPLSM. A representative result of electrically evoked $[Ca^{2+}]_i$ transients that were recorded at the graft-host border following transplantation of low-density bone marrow cells is illustrated in Figure 4. A cluster of small ($< 10 \mu\text{m}$ diameter), round, EGFP-expressing, i.e., donor-derived, cells were observed to be sandwiched between host cardiomyocytes. Periodic $[Ca^{2+}]_i$ transients were readily visible in the surviving host myocytes during remote point stimulation, but were undetectable in the transplanted cells. The line-scan image which was obtained by repeatedly scanning along the blue line in panel A, and the time plots of EGFP and rhod-2 fluorescence intensities combined (panel C) confirm the absence of periodic $[Ca^{2+}]_i$ changes in the donor cells. Screening of hundreds of donor cells located along the graft/host junction failed to reveal a single EGFP-expressing cell exhibiting electrically evoked $[Ca^{2+}]_i$ transients. Identical results were obtained with c-kit enriched cells and hematopoietic stem cells when transplanted into infarct border zone hours after onset of ischemic injury. Collectively, these results indicate that bone marrow-derived cells lack the capacity to develop $[Ca^{2+}]_i$ transients in response to electrical membrane excitation, and consequently cannot function as cardiomyocytes. Our findings are consistent with the notion that bone marrow-derived cells have only limited capacity to transdifferentiate into cardiomyocytes, in agreement with previous results that were obtained through molecular and structural analyses [20,21]. Nevertheless, despite the lack of significant transdifferentiation of the engrafted bone marrow cells into cardiomyocytes, the contractile function of infarcted hearts improved to a significant degree. Results of clinical trials were overall less persuasive than those reported in preclinical animal studies, with the exception of a more recent phase I clinical trial [22]. Given the lack of cardiomyogenic potential of bone marrow cells applied to the ischemically injured myocardium, the observed increase in cardiac function must result from indirect, most likely paracrine, effects of the bone marrow cell grafts imparted upon the surviving host myocardium, including anti-inflammatory, pro-angiogenic and anti-apoptotic effects.

In additional studies, we examined the potential of cardiac-resident phase bright cells to functionally integrate with the host myocardium following their transplantation into ischemically injured hearts [23]. Explant-derived cells were prepared from cardiac explant cultures of transgenic mice ubiquitously expressing EGFP and transplanted into the peri-infarct border zone of syngeneic non-transgenic mice following coronary artery ligation. TPLSM imaging 3 weeks later revealed the presence of donor cells in the host myocardium, but electrical stimulation (both remote point stimulation and field stimulation) failed to elicit

$[Ca^{2+}]_i$ transients in any of the donor-derived cells scanned, whereas they were readily inducible in the remote host myocardium and in surviving host cardiomyocytes within the peri-infarct zone. Thus, although donor cells survived for at least 3 weeks following engraftment, they lacked elements required for excitation-contraction coupling, rendering this approach unsuitable for cell-based myocardial regeneration strategies.

All findings are summarized in Table 1.

Future

Sources of cells for cellular transplantation also include cardiomyogenic stem cells that are derived from embryonic stem cells [24,25] and reprogrammed adult-derived cells with embryonic stem cell-like cardiomyogenic activity (i.e., induced pluripotent stem cells [26], parthenogenetically derived stem cells [27], and spermatogonially derived stem cells [28,29]). Although in some instances their stable engraftment into the ischemically injured heart muscle has been documented and circumstantial evidence has been provided for the ability of embryonic stem cell-derived cardiomyocytes to electrically couple with the host myocardium [30], future studies will need to quantitatively define the propensity of these clinically promising donor cell types to stably and functionally couple with the recipient myocardium following their intracardiac delivery. We believe a TPLSM-based assay to be a useful tool to directly address these important issues.

Acknowledgments

This study was supported by NIH grant (RO1HL075165 to M. R.) and the Riley Children's Foundation.

References

1. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J. Evidence for cardiomyocyte renewal in humans. *Science*. 2009; 324:98–102. [PubMed: 19342590]
2. Hsieh PC, Segers VF, Davis ME, MacGillivray C, Gannon J, Molkentin JD, Robbins J, Lee RT. Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nat Med*. 2007; 13:970–974. [PubMed: 17660827]
3. Oka T, Komuro I. Molecular mechanisms underlying the transition of cardiac hypertrophy to heart failure. *Circ J*. 2008; 72(Suppl A):A13–16. [PubMed: 18772527]
4. Laflamme MA, Zbinden S, Epstein SE, Murry CE. Cell-based therapy for myocardial ischemia and infarction: pathophysiological mechanisms. *Annu Rev Pathol*. 2007; 2:307–339. [PubMed: 18039102]
5. Rubart M, Field LJ. Cardiac regeneration: repopulating the heart. *Annu Rev Physiol*. 2006; 68:29. [PubMed: 16460265]
6. Centonze VE, White JG. Multiphoton excitation provides optical sections from deeper within scattering specimens than confocal imaging. *Biophys J*. 1998; 75:2015–24. [PubMed: 9746543]
7. Rubart M. Two-photon microscopy of cells and tissue. *Circ Res*. 2004; 95:1154–66. [PubMed: 15591237]
8. Young PA, Clendenon SG, Byars JM, Decca RS, Dunn KW. The effects of spherical aberration on multiphoton fluorescence excitation microscopy. *J Microsc*. 2011; 242(2):157–65. [PubMed: 21118240]
9. Young PA, Clendenon SG, Byars JM, Dunn KW. The effects of refractive index heterogeneity within kidney tissue on multiphoton fluorescence excitation microscopy. *J Microsc*. 2011; 242(2):148–56. [PubMed: 21118239]
10. de Monvel JB, Scarfone E, Le Calvez S, Ulfendahl M. Image-adaptive deconvolution for three-dimensional deep biological imaging. *Biophys J*. 2003; 85:3991–4001. [PubMed: 14645088]

11. Rubart M, Wang E, Dunn KW, Field LJ. Two-photon molecular excitation imaging of Ca²⁺ transients in Langendorff-perfused mouse hearts. *Am J Physiol Cell Physiol*. 2003; 284:C1654–68. [PubMed: 12584115]
12. Biermann M, Rubart M, Moreno A, Wu J, Josiah-Durant A, Zipes DP. Differential effects of cytochalasin D and 2,3 butanedione monoxime on isometric twitch force and transmembrane action potential in isolated ventricular muscle: implications for optical measurements of cardiac repolarization. *J Cardiovasc Electrophysiol*. 1998; 9(12):1348–57. [PubMed: 9869534]
13. Rubart M, Pasumarthi KB, Nakajima H, Soonpaa MH, Nakajima HO, Field LJ. Physiological coupling of donor and host cardiomyocytes after cellular transplantation. *Circ Res*. 2003; 92(11):1217–24. [PubMed: 12730096]
14. Rubart M, Soonpaa MH, Nakajima H, Field LJ. Spontaneous and evoked intracellular calcium transients in donor-derived myocytes following intracardiac myoblast transplantation. *J Clin Invest*. 2004; 114(6):775–83. [PubMed: 15372101]
15. Roell W, Lewalter T, Sasse P, Tallini YN, Choi BR, Breitbach M, Doran R, Becher UM, Hwang SM, Bostani T, von Maltzahn J, Hofmann A, Reining S, Eiberger B, Gabris B, Pfeifer A, Welz A, Willecke K, Salama G, Schrickel JW, Kotlikoff MI, Fleischmann BK. Engraftment of connexin 43-expressing cells prevents post-infarct arrhythmia. *Nature*. 2007; 450(7171):819–24. [PubMed: 18064002]
16. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001; 410(6829):701–5. [PubMed: 11287958]
17. Strauer BE, Brehm M, Zeus T, Köstering M, Hernandez A, Sorg RV, Kögler G, Wernet P. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation*. 2002; 106(15):1913–8. [PubMed: 12370212]
18. Assmus B, Schächinger V, Teupe C, Britten M, Lehmann R, Döbert N, Grünwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation*. 2002; 106(24):3009–17. [PubMed: 12473544]
19. Scherschel JA, Soonpaa MH, Srour EF, Field LJ, Rubart M. Adult bone marrow-derived cells do not acquire functional attributes of cardiomyocytes when transplanted into peri-infarct myocardium. *Mol Ther*. 2008; 16(6):1129–37. [PubMed: 18431364]
20. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature*. 2004; 428(6983):664–8. [PubMed: 15034593]
21. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature*. 2004; 428(6983):668–73. [PubMed: 15034594]
22. Penn MS, Ellis S, Gandhi S, Greenbaum A, Hodes Z, Mendelsohn FO, Strasser D, Ting AE, Sherman W. Adventitial delivery of an allogeneic bone marrow-derived adherent stem cell in acute myocardial infarction: phase I clinical study. *Circ Res*. 2012; 110(2):304–11. [PubMed: 22052917]
23. Shenje L, Guerin C, Pritchard CA, Ang K, Rubart-von der Lohe M, Soonpaa MH, Field LJ, Galinanes M. Lineage Tracing of Cardiac Explant Derived Cells. *PLoS ONE*. 2008; 3:e1929. [PubMed: 18414652]
24. Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. *J Clin Invest*. 1996; 98(1):216–24. [PubMed: 8690796]
25. Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S, O'Sullivan C, Collins L, Chen Y, Minami E, Gill EA, Ueno S, Yuan C, Gold J, Murry CE. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol*. 2007; 25(9):1015–24. [PubMed: 17721512]
26. Mauritz C, Schwanke K, Reppel M, Neef S, Katsirntaki K, Maier LS, Nguemo F, Menke S, Hausteiner M, Hescheler J, Hasenfuss G, Martin U. Generation of functional murine cardiac

- myocytes from induced pluripotent stem cells. *Circulation*. 2008; 118:507–517. [PubMed: 18625890]
27. Didié M, Christalla P, Schwoerer AP, Ehmke H, Scherschel JA, Rubart M, Soonpaa MH, Field LJ, Eschenhagen T, Zimmermann WH. Derivation of functional cardiomyocytes from parthenogenetic stem cells. *Circulation*. 2008; 118:S428, Abstract 3457.
 28. Guan K, Wagner S, Unsöld B, Maier LS, Kaiser D, Hemmerlein B, Nayernia K, Engel W, Hasenfuss G. Generation of functional cardiomyocytes from adult mouse spermatogonial stem cells. *Circ Res*. 2007; 100(11):1615–25. [PubMed: 17478732]
 29. Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature*. 2006; 440(7088):1199–203. [PubMed: 16565704]
 30. Kehat I, Khimovich L, Caspi O, Gepstein A, Shofti R, Arbel G, Huber I, Satin J, Itskovitz-Eldor J, Gepstein L. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. *Nat Biotechnol*. 2004; 22(10):1282–9. [PubMed: 15448703]

Figure 1.

TPSLM $[Ca^{2+}]_i$ imaging in Langendorff-perfused mouse heart. **A**, Frame-mode image obtained from a rhod-2 – loaded Langendorff-perfused mouse heart in the presence of cytochalasin D (50 μ M). The heart was electrically stimulated at a remote site when the scan was approximately halfway across the cardiomyocytes in the field of vision. Wavelength for two-photon illumination was 810 nm and rhod-2 emission was collected between 560 and 650 nm. Arrowheads denote endothelial cell nuclei. **B**, Line-scan image from the heart in **A**. The red line in panel **A** was repeatedly scanned at a rate of 32 Hz during remote point stimulation at 1 and 2 Hz and the fluorescence intensities along each line were stacked vertically. The scan line traversed three neighboring cardiomyocytes. **C**, Plots of spatially averaged fractional rhod-2 fluorescence (F/F_0) changes as a function of time for each of the three cardiomyocytes shown in **B**. **D**, Rate-dependent $[Ca^{2+}]_i$ transient shortening. For comparison of the $[Ca^{2+}]_i$ transient kinetics among the three cardiomyocytes, fluorescence intensities were normalized to their respective peaks. From [11]

Figure 2.

Short-term exposure to cytochalasin D does not alter spatial profiles of action potential-evoked $[\text{Ca}^{2+}]_i$ rises in *in situ* ventricular cardiomyocytes. **A**, Confocal line-scan images of action potential-evoked changes in fluo-4 fluorescence intensity in an isolated murine ventricular cardiomyocyte in the absence of cytochalasin D (left panel) and a ventricular cardiomyocyte in a Langendorff-perfused mouse heart in the presence of 50 $\mu\text{mol/L}$ cytochalasin D. Images were obtained by taking repeated scans along the same lines parallel to the longitudinal axes of the cells and stacking the intensity of each scan line vertically. Electrical stimulation resulted in a rapid and relatively uniform increase in fluorescence along the scanned lines, indicating highly coordinated Ca^{2+} release from the sarcoplasmic reticulum. **B**, Time course of normalized fluorescence from the two cells shown in **A**. Fluorescence intensities (F) of all pixels along the scan lines were averaged, normalized to F at baseline (F₀), and plotted as a function of time. Note the similarity in the upstroke kinetics between the two cells

Figure 3.

Physiological coupling of cardiomyocyte grafts following intracardiac transplantation. **A**, Frame-mode image taken from a rhod-2 – loaded mouse heart carrying an EGFP-expressing fetal cardiomyocyte graft. Host cardiomyocytes appear yellow due to overlap of green EGFP and red rhod-2 fluorescence. Periodic, ripple-like wave fronts reflect action potential-evoked $[Ca^{2+}]_i$ transients. Stimulation frequency, 2 Hz. **B**, Line-scan image obtained by repeatedly scanning along the white line in **A** and stacking the lines vertically. **C**, Time plots of spatially averaged rhod-2 and EGFP fluorescence during pacing at 1 and 2 Hz for cells 1 and 2 of panel **B**. **D**, Normalized *in situ* $[Ca^{2+}]_i$ transients of a donor cardiomyocyte and two neighboring host cardiomyocytes. From [13]

Figure 4.

Spatial micro-heterogeneity of calcium handling at the skeletal/cardiac muscle junction. **A**, Frame-mode images obtained during sinus rhythm (left panel) and electrical field stimulation from a rhod-2 – loaded mouse heart carrying an EGFP-expressing skeletal myotube graft. Scale bar, 20 μ m. **B**, Frame-mode (*a*, *b* and *c*) and line-scan mode (*d*, *e* and *f*) images taken from the skeletal muscle-myocardium border following transplantation of EGFP-expressing skeletal myoblasts. Line-scan images were obtained by repeatedly scanning along the white lines in *a*, *b* and *c* during remote point stimulation at 2 Hz. Normalized Ca transients (*g*–*i*) for the donor-derived and host myocytes shown in *a*–*f*. From [14] with permission.

Figure 5.

Bone marrow-derived cells do not acquire functional attributes of cardiomyocytes following engraftment in ischemically injured myocardium. **A**, Frame-mode image taken from the graft-host border. The heart was loaded with rhod-2. Red (rhod-2) and green (EGFP) signals were superimposed. Host cardiomyocytes and donor-derived cells are apparent. The heart was paced at 4 Hz at a remote site. The white bar demarcates the position of the line-scan mode data acquisition. Asterisks denote host cardiomyocytes with $[Ca^{2+}]_i$ transients. **B**, Stacked line-scan image of the region in **A** demarcated by the blue line. **C**, Spatially integrated changes in rhod-2 and EGFP fluorescence for one host cardiomyocyte and one juxtaposed donor-derived cell. The fluorescence intensities across the entire cell widths were averaged. From [19].

Table 1

Extent of functional donor-host cell coupling following cellular transplantation into uninjured or infarcted mouse heart

Donor cell type	Recipient heart	Extent of donor-host cell coupling based on TPLSM $[Ca^{2+}]_i$ imaging	Reference
EGFP-expressing fetal cardiomyocytes	uninjured	All donor cells in physical contact with host myocytes were coupled	[13]
EGFP-expressing primary myoblasts	uninjured	A small fraction of donor-derived cells arising from donor-host myocyte fusion events were coupled	[14]
EGFP-expressing adult bone marrow- derived cells	infarcted	None of >3000 donor cells imaged were coupled	[19]
EGFP-expressing cardiac explant- derived cells	infarcted	None of 50 donor-derived cells imaged were coupled	[23]