440-Pos Doxorubicin Decreases Creatine Transport In Hl-1 Cardiac Myocytes Expressing The Human Creatine Transporter

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Doxorubicin (DXO) is a widely used chemotherapeutic agent commonly used to treat leukemia as well as solid tumors. A major side-effect of its administration is cardiotoxicity that may cause cardiomyopathy and heart failure (HF) in a significant number of patients (Minotti, 2004 Pharmacol Rev *56*, 185–229). The molecular mechanisms leading to HF in this setting are poorly understood. DXO circulating at therapeutic levels in isolated perfused heart preparations alters high-energy phosphate metabolism via the creatine (Cr) energy shuttle. The Cr kinase isoform distribution is shifted, and enzymatic activity is decreased (Tokarska -Schlattner, 2005 Am J. Physiol Heart Circ. Physiol *289*, H37–H47). We have characterized the effect of DXO treatment on Cr transport in HL-1 murine cardiac cells expressing the human Cr transporter (CrT) protein. We demonstrate that DXO diminishes Cr transport in a dose and time dependent manner. A statistically significant (n=4 ANOVA, $p<0.05$ Fisher LSD) decrease in Cr transport is detectable after 24 hrs of incubation with 50nM DXO, a concentration that is forty fold less than the 2 μM plasma concentration typically achieved after administration of a bolus of DXO during the course of chemotherapy. Kinetic analysis reveals that incubation with 100nM DXO causes a statistically significant increase in Km (45.5 ± 1.7 vs 37.7 ± 1.85 μM in controls, n=4, $p<0.05$, t-test), and tends to decrease Vmax (6.07± 0.7 vs 4.7 ± 0.48 nmol/mg protein in controls, $p = 0.16$). The decrease in Vmax is mirrored by a decrease in abundance of total CrT protein. We conclude that a decrease in Cr transport may be responsible for the bioenergetic derangements observed in DXO-associated heart failure.

Intercellular Communication & Gap Junctions

441-Pos The Relevance of Non-Excitable Cells for Cardiac Pacemaker Function

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Aging and sinus node disease induce an increased ratio of cardiomyocytes to fibroblasts in the sinoatrial node and it has been demonstrated that cardiomyocyte spontaneous activity can be attenuated when intercellularly coupled to and depolarized by fibroblasts. Our goal was to determine if the mere physical separation of neighboring cardiomyocytes by interspersed fibroblasts modulates spontaneous activity and conduction velocity (υ) independent of their depolarizing effect.

The spontaneous activity and υ in multicellular cardiomyocyte preparations (HL-1 monolayers) were monitored with microelectrode arrays. Myocyte+fibroblast (WT- or Cx43(−/−)-fibs) co-cultures were established by either 'mixing' the fibroblasts between the cardiomyocytes or by 'layering' them onto confluent cardiomyocyte monolayers. This way the effect of fibroblast infiltration could be separated from their effect on cardiomyocyte V_m. WT-fibs reduced the beating frequency of HL-1 cells in mixed (62±5%; n=13) and layered (63±4%;n=10) preparations. HL-1+Cx43(−/−)-fibs co-cultures exhibited decreased heterocellular coupling and attenuated cardiomyocyte V_m depolarization; however, the decreased beating frequency persisted in 'mixed' cultures (61±6%; n=30). Only in layered HL-1+ Cx43(−/−)-fibs co-cultures the fibroblasts' effect on spontaneous activity (7±2%;n=16) could be prevented. In contrast, although υ in mixed and layered cultures correlated negatively with the amount of WT-fibs, the decrease of υ in both mixed and layered co-cultures of HL-1+Cx43(−/−)-fibs was significantly attenuated. Comparable to uncoupled cardiomyocyte preparations, in both mixed co-cultures of HL-1+WT and HL-1+Cx43(−/−)-fibs, the co-efficient of the inter-beat interval (C_{IBI}) was significantly increased (694±198% and 587±112%). However, C_{IBI} remained comparable to control in 'layered' HL-1+WT-fibs or +Cx43(−/−)-fibs cultures. The data demonstrate that fibroblasts modulate cardiomyocyte excitability through intercellular coupling and cardiomyocyte depolarization; however, their major effect on the spontaneous activity is mediated by separating neighboring cardiomyocytes, which is, for the heterocellular resistances tested, independent from the intercellular coupling established.

442-Pos Movement of M3 Helices During Gating of Cx43 Gap-Junctional Hemichannels by PKC-Mediated Phosphorylation

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Board B274

The decrease in permeability to large solutes through gap-junctional channels (GJC) and hemichannels (GJH) in response to lowering pH and phosphorylation is believed to occur through a ball-and-chain mechanism, with the C-terminal domain (CTD) acting as the ball. It has been proposed that decreases in cytoplasmic pH and phosphorylation cause a movement of the CTD with association to other intracellular domains, which results in a decrease in the effective channel pore size. Our goal was to determine whether a ball-and-chain mechanism could explain the regulation of Cx43 GJH permeability by PKC-mediated phosphorylation. We studied purified and reconstituted GJH formed by Cx43 variants that contain only one cysteine residue at predetermined positions (I156C, V164C, V167C and a Cys added at the C-terminal end), for labeling with acceptors probes (fluorescein, ATTO-465 or ABD). We then measured Angstrom distances between probes in homologous residues in different

subunits of the GJH using luminescence resonance energy transfer, using Tb^{3+} as donor. Our data show that phosphorylation by PKC produces movements of the CTD, but also a large decrease (4–7 Angstrom) in the distance between the M3 helices in different subunits. The results indicate that a simple ball-and-chain mechanism cannot explain the gating of Cx43 GJH by PKC-mediated phosphorylation, and that a significant re-arrangement of pore helices takes place during gating by PKC-mediated phosphorylation.

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443-Pos Micropatterning of Costimulatory Ligands Enhances T Cell Function

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Microscale organization of signaling complexes within the immune synapse (IS) has emerged as a powerful modulator of T cell function. This report examines how the distribution of TCR and CD28 complexes modulates T cell costimulation, using patterns inspired by natural IS structures. Arrays of multicomponent patterns of activating antibodies to CD3 and CD28 were created on planar substrates using successive rounds of microcontact printing. In a colocalized pattern, CD3 and CD28 antibodies were presented together in a single 2 micrometer feature. In a separate, segregated pattern, CD28 antibodies were moved to the IS periphery, forming a constellation of four 1 micrometer circles spaced 4 micrometers apart and surrounding a central, 2 micrometer anti-CD3 feature. These patterns thus modulate the geometry of molecular complexes while maintaining the same amount of ligand within the IS. ICAM-1 was adsorbed to the remaining surface, completing this trifunctionalized substrate.

IL-2 secretion by naïve CD4+ T cells interacting with these costimulation arrays was assayed over 6-hours as a measure of T cell function. Segregation of the anti-CD28 signal significantly enhanced IL-2 secretion in comparison to the colocalized pattern, demonstrating that these cells are able to recognize and respond to micrometer-scale changes in ligand presentation. Inhibition of the PKB/Akt signaling pathway reduced IL-2 secretion on both patterns to levels that were similar to each other, but greater than that on surfaces containing anti-CD3 alone. This pathway could thus completely account for the ability of T cells to respond to pattern geometry. Finally, experiments using variations on the basic costimulation patterns suggest that distribution of CD28 ligands, rather than its relation to CD3 signal, is the primary determinant of T cell costimulation. The full impact, and mechanisms, of signal segregation on T cell activation are currently under investigation.

444-Pos The N-Terminus of Connexin37 is α-Helical and Mutations that Disrupt the α-Helix also Disrupt Channel Gating

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The cytoplasmic N-terminal domain of connexins has been implicated in connexin trafficking/assembly and channel gating. We prepared a synthetic peptide corresponding to the first 23 amino acids of human connexin37 (Cx37) and determined its structure by NMR. To assess the importance of this structure in trafficking and gating, the behavior of wild type Cx37 (WTCx37) tagged at its C-terminus with GFP was compared with that of site-directed mutants that would disrupt the N-terminal structure. Formation of gap junction plaques was assessed in transiently transfected HeLa cells by fluorescence microscopy, and formation of functional hemichannels was assessed using two-microelectrode voltage clamp in cRNA injected *Xenopus* oocytes. The NMR studies showed that the Cx37 N-terminal peptide was predominantly α-helical (especially between Gly-5 and Glu-16). Therefore, we produced Cx37 mutants containing substitutions of prolines or glycines designed to disrupt the α-helix (Cx37L10P/Q15P and Cx37L10G/Q15G). WTCx37 and both substitution mutants reached the plasma membrane and formed gap junction plaques in transfected HeLa cells. Hemichannel currents were observed after expression of WTCx37 in *Xenopus* oocytes, but neither the Pro nor the Gly substitution construct produced hemichannel currents. A moderate reduction in the magnitude of the induced currents was detected in *Xenopus* oocytes injected with 1:1 cRNA mixtures of WTCx37 and Cx37L10G/Q15G suggesting that the mutant acted as a weak dominant-negative. These results demonstrate that the N-terminus of Cx37 is α-helical and that this structure is required for function, but not for trafficking to the plasma membrane or formation of gap junction plaques.

445-Pos Structural Analysis of Full Length and N-terminus Deleted Connexin 26 Gap Junction Channels Exhibits a Significant Difference in the Plug Density

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We recently reported the three dimensional structure of a mutant human connexin26 (Cx26M34A) by electron cryo-crystallography

that revealed a significant density in the vestibule of the channel pore suggestive of a physical plug blocking the channel (Oshima *et al.* (2007) *PNAS*). Electrophysiology of the Cx26M34A revealed that the open probability of the mutant was significantly reduced and dye transfer was also eliminated. However, based on the normal voltage responses of the residual currents, this closed state may not represent voltage gating. While previous studies suggest that the connexin N-terminus may lie in the aqueous pore vestibule (Purnick *et al*. (2000) *Biophys. J*., Purnick *et al*. (2000) *Arch. Biochem. Biophys*.), it is not clear if this part of the sequence contributes to the plug.

To further address this issue, we constructed a deletion mutant of Cx26M34A missing amino acids 2–7 (Cx26M34Adel2-7), expressed it in baculovirus infected Sf9 cells and crystallized this mutant in the same crystal form as previously reported. A projection map of Cx26M34Adel2-7 at 10 Å resolution showed a pore with significantly decreased density. The difference map between Cx26M34A and Cx26M34Adel2-7 projection maps revealed a strong peak (~4.5 sigma) at the position corresponding to center of the pore. An initial 3D map of Cx26M34Adel2-7 determined at 10 Å resolution revealed the plug density is drastically reduced in this new structure as compared to Cx26M34A channel while the overall tertiary structure of the channel is retained. These results suggest that the N-terminus may contribute to the conformation of the plug domain. A full 3D structure determination and functional analysis of Cx26M34Adel2-7 are in process to elucidate the role that the N-terminus plays in gating of gap junction channels.

446-Pos Study of Cell-cell Signaling in 3D Bacterial Arrays Assembled using Optical Tweezers

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Key for full understanding the paracrine signaling is the control of the local environment and position of each cell participating in signaling. Here, we report on 3D cell-to-cell signaling experiments that use two types of genetically manipulated E.coli: the first type is a sender that uses the lac promoter controlling the production of red fluorescence protein and luxI, which catalyzes the production of acyl-homoserine lactone (AHL), a signaling molecule. The second type is a receiver that in response to AHL expresses GFP-LVA regulated by lux operon. The threshold value of AHL concentration for switching on lux promoter in receiver cells is ~10 nM. Using optical traps we have assembled 3D arrays of these cells placed in various locations. Assembly of each array is done in a microfluidic chip that allows full control over the entire assembly. After cells are placed in desired location, the positions of the cells in the array are fixed permanently with the nanometer precision in a photopolymerizable, biocompatible hydrogel.

By placing array of cells in 3 different planes separated by 10 um and by varying the flow rate at each plane in our microfluidic setup we show that signaling can be locally controlled. In contrast to recent studies in bulk this experiment unveils the stochastic nature of cell-to-cell signaling. We monitored the time dependent production rate of the signaling molecule by sender and response of the receiver via fluorescence protein reporters mentioned above. We also have simulated this system, by using a diffusion/convection mass transport equation coupled to a series of mass-action kinetics equations describing the protein production in the bacterial cells. Using the simulation, parameters of each of the individual cells were extracted using a best fit algorithm to match the fluorescent data.

447-Pos ATP release through Connexin 32 hemichannels

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Board B279

Connexins are the proteins responsible of forming gap junctions. Six subunits are necessary to form a hemichannel or connexon anchored in the plasma membrane. Two hemichannels of adjacent cells form a gap junction. At present, there are, at least, 20 isoforms of connexins described. Particularly, mutations in Connexin 32 (Cx32) have been associated to the X-linked form of Charcot-Marie-Tooth disease, a neurodegenerative illness affecting the peripheral nervous system. The Cx32 is expressed in Schwann cells, in the paranodal zones. Using *Xenopus* laevis oocytes to express Cx32 hemichannels, we have monitored simultaneously the release of ATP and the ionic currents. ATP in the medium was detected with luciferin-luciferase reaction and the ionic currents were recorded under two electrode voltage clamp. Depolarization of oocytes expressing Cx32, from −40mV to +80mV induced an outward current and at the end of the pulse, a transient inward tail current was detected. A transitory peak of ATP release was associated with this tail current. Using Cx32 tranfected HeLa cells, we captured the luminescence due to ATP release, when cells were under a hypotonic solution containing luciferin-luciferase mixture. Isotonic solution was 280 mOsm and hypotonic solution was 150 mOsm. We also studied the release of ATP in Schwann cell cultures and again the hypotonic conditions induced a rapid increase of extracellular ATP. Finally, in teased nerve fibres, isolated from the mouse sciatic nerve, we also recorded the release of ATP in hypotonic conditions and interestingly we found the maximal release of ATP in the paranodal zones of Schwann cells. Considering that Cx32 is also found in this part of the Schwann cells, we suggest that ATP is released from Schwann cells through Cx32.

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448-Pos A 3D Mathematical Model Of Intercellular Ca^{2+} Signaling In Normal And Diabetic Mouse Detrusor Muscle

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Bladder voiding by detrusor muscle contraction depends on specific agonists activating calcium-mobilizing and calcium-sensitizing pathways. We here describe a 3D stochastic model and an associated computer program of intercellular Ca^{2+} wave spread that permits individual change of components, allowing comparison of simulations in normal and diabetic rat detrusor muscle. In this model, restricted to 3 cell layers in which the myocytes are randomly distributed with a given density in 7x7 staggered rectangular grids, the agonists released from a single nerve terminal and from urothelium diffuse and are degraded both in the bulk solution and at muscle cell surfaces. Agonist binding to ionotropic receptors induces Ca^{2+} influx from the bulk solution, while binding to metabotropic receptors initiates formation of the second messenger, IP_3, that triggers release of Ca^{2+} from endoplasmic reticulum stores. The messenger is degraded within the cytosol and diffuses to neighboring cells through gap junction channels. Variability of ICWs is provided by randomly selecting values characterizing agonist release, extracellular compartment, individual cells and cell interconnections within experimentally determined intervals from healthy and diabetic rats. Such modeling is expected to be useful for testing phenomenological hypotheses and in understanding consequences of alteration of system components under experimental or pathological conditions.

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449-Pos Reversal Of Gap Junctions Remodeling In Rabbit Hearts Following Infarct Exclusion Surgery

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Board B281

Objectives: Left ventricular (LV) chronic myocardial infarction (CMI) impairs pump function and provides substrates for increased risk of lethal arrhythmia. Exclusion of the infarct can be achieved through LV reconstruction (LVR) surgery to restore chamber geometry and hemodynamic function. The impact of such intervention on subsequent electrical and molecular remodeling is however less studied. We investigated Cx43 expression and phosphorylation in LVR rabbit model to assess post-LVR reversal or attenuation of Cx43 remodeling due to CMI.

Methods: Transmural LV infarct was created by ligation of the major branch of left coronary artery. LVR surgery was performed on four rabbits to exclude the infarct. Using Western blotting and immunoconfocal microscopy, we measured Cx43 level and phosphorylation in LV boarder zone (BZ) and in area away from BZ from control, CMI, and LVR groups.

Results: In CMI BZ, total Cx43 was significantly reduced compared to the area away from BZ within the same group and to the similar region in the control group. Immunolabeling revealed lateralization of Cx43 outside the end-to-end junction. In LVR BZ, Cx43 expression was still downregulated compared to the control group. However, compared to CMI BZ, Cx43 increased substantially and Cx43 dephosphorylated at ser-368 was markedly reduced. Although Cx43 retained lateralization, it was also abundant at the end-to-end junction.

Conclusions: In CMI BZ, there is a decrease in Cx43 expression and phosphorylation and increase in lateralization. Increased total level of Cx43 and reduction of dephosphorylated Cx43 in LVR BZ signify reversal of Cx43 due to increase in the amount of functional, phosphorylated Cx43 and more homogeneous distribution profile of Cx43 across the myocardium than CMI. These may result in faster and more uniform electrical conduction which could contribute to possible reduction of arrhythmogenesis in LVR hearts.

450-Pos Isolation of Pannexin1 and Pannexin2 Channels Reveals Structural Similarity to Gap Junction Hemichannels

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Board B282

Pannexins are a family of proteins that share a common folding topology with connexins, the components of gap junction channels. They are predicted to have four transmembrane regions, two extracellular plus one intracellular loop and intracellular N and C terminals. The human pannexin family consists of three members: Pannexin1 (Panx1, 47.6 kDa), Pannexin2 (Panx2, 73.3 kDa) and Pannexin3 (Panx3, 44.7 kDa). The three murine pannexins have similar molecular weights to their human homologs. Functional data demonstrated that these channels act as mechanosensitive ATP channels. Recent cross-linking data showed that Panx1 forms a hexamer and is N-glycosylated, but does not form canonical gap junctions. Instead, these channels exist as hexamers (pannexons) dispersed throughout the plasma membrane. However, no direct structural data exist for the pannexin molecular structure.

Here, we applied methods for purification and structural analysis that we developed for connexin-based gap junctions to rat Panx1 and Panx2. By electron microscopy (EM), we have been able to identify cellular membranes that clearly show pannexin channels when isolated from MDCK cells stably expressing rPanx1 or rPanx2. Immunolabeling by secondary antibodies with colloidal gold-conjugated for EM and western blots for protein analysis confirmed that rPanx1 or rPanx2 form the doughnut-like structures visible in our membrane preparations. As a negative control, we analyzed membranes from parental HeLa cells that do not endogenously express Panx1 or Panx2 and are communication deficient. These membranes did not contain any channel structures, failed to label with immunogold and lacked pannexin bands on corresponding western blots. In addition, purified pannexons from both rPanx1-His6-baculovirus or rPanx2-His6-baculovirus infected Sf9 cells confirmed the doughnut-like structures observed in mammalian membranes and represent useful specimens for further structural analysis by electron cryo-microscopy.

451-Pos Assessing Cadherin-Cadherin Binding Dynamics in Living Cells Using Förster Resonance Energy Transfer (FRET)

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Board B283

Cadherins are a major family of calcium-dependent cell-cell adhesion molecules that are found at cellular junctions and neuronal synapses. Classic cadherins interact via homotypic adhesion_in a cis-dimer interaction within each membrane as well as a trans-dimer (across junction) interaction between the cis dimers. To monitor directly the binding dynamics, we have developed a Förster Resonance Energy Transfer (FRET) reporter system for visualizing homophilic interactions of cadherin in living cells. We created N-cadherin fusion proteins with an intramolecular fluorescent protein insertion (cerulean, donor and venus, acceptor) using a transposon-mediated insertion method. We generated fluorescently labeled N-cadherin that is functional, exhibits fluorescence of sufficient intensity for FRET, mimics the cellular localization of endogenous N-cadherin, and interacts with the correct binding partner, β-catenin. In heterologous cells, we can probe within cell or across junction cadherin interactions when individual cells are co-transfected and express both constructs (cis) or neighboring cells express either the donor or acceptor (trans). The dynamics of Ca^{2+}-dependent cadherin associations can be monitored by manipulating extracellular Ca^{2+}. Since cadherins play a critical role in synaptic plasticity, we will use the FRET reporter system in neurons to directly test whether synaptic activity regulates the adhesive strength of cadherin interactions across synapses. FRET will be used to detect cadherin interactions between pre- and postsynaptic cells during synaptic activity and varying extracellular Ca^{2+}concentrations. These experiments will test our hypothesis that cadherins act as extracellular calcium detection system to coordinate synaptic plasticity across the synapse.

Calcium Signaling Pathways

452-Pos Ca^{2+} Store Depletion Triggers A Dynamic, Physical Coupling Of Stim1 To Orai1

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Board B284

Store-operated Ca^{2+} entry (SOCE) is a physiologically important process that is triggered by the depletion of intracellular Ca^{2+} stores. Recently, STIM1 an endoplasmic reticulum (ER) protein and ORAI1, which is thought to act as the channel unit in the plasma membrane (PM), were identified as essential components of the classical calcium-release-activated calcium (CRAC) current. STIM1 with an EF hand motif in the lumen of the ER, senses the Ca^{2+} concentration and redistributes after store depletion into punctae in regions tight beneath the PM. Here we demonstrate by confocal Förster Resonance Energy Transfer (FRET) microscopy that this redistribution results in a dynamic, physical coupling of STIM1 to ORAI1 that culminates in the activation of Ca^{2+} entry. FRET imaging of living cells provided insight in the time-dependence of crucial events of this signalling pathway comprising Ca^{2+} store depletion, STIM1 multimerization and STIM1-ORAI1 interaction and furthermore resolved a significant time lag between STIM1-STIM1 and STIM1-ORAI1 interactions. Store refilling reduced both STIM1 multimerization as well as STIM1-ORAI1 interaction suggesting a reversible process, which is specifically linked to the loading state of the ER. The cytosolic STIM1 C-terminus itself was able, in vitro as well as in vivo, to physically associate with ORAI1 and to stimulate channel function without store depletion indicating a crucial role of this domain in the activation mechanism. Our experiments do not exclude that CRAC current activation may well occur with the help of auxiliary components. Further elucidation of such components involved in fine tuning of the STIM1-ORAI1 coupling process will hopefully widen the repertoire for manipulation of this key mechanism by the development of both specific inhibitors as well as activators.

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453-Pos Shear-stress Induced Ca^{2+} Transients in Ventricular Myocytes are Associated With Rapid Decline of the Mitochondrial Ca^{2+} Content

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Board B285

In cardiomyocytes, we have found that brief "puffs" of solution (shear-stress) trigger cytosolic Ca^{2+}-transients that are independent of SR Ca^{2+} release and display pharmacology indicative of a mitochondrial origin. To investigate mitochondrial Ca^{2+}-signaling, we used rapid confocal fluorescence imaging of cells stained with the potentiometric dye, TMRE or the Ca^{2+}-sensitive dye, rhod-2. The images showed the mitochondria as longitudinal lines with a separation of 1-2 μm and a regular striation pattern at ~2 μm. During electrical stimulation, KCl-depolarization, "puffs", and exposure to caffeine or mitochondrial blockers (FCCP or CCCP), the images revealed not only a change in the overall fluorescence intensity, but also redistribution between the mitochondrial and cytoplasmic compartments. To eliminate contraction artifacts, we analyzed the images based on their Fourier-transforms, where the typical mitochondrial pattern was represented by transverse and sarcomeric harmonics. Changes in the TMRE-images displayed different time-course for average fluorescence intensity vs. harmonics, suggesting that this dye increases its brightness when redistributed from the