Direct measurement of myofilament calcium in living cardiomyocytes.

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WORD COUNT 1498

Abstract:

Subcellular calcium indicators applied to cardiomyocytes reveal an orchestra of intracellular sources interacting during every heartbeat. Here we describe a myofilament-restricted calcium indicator which enables visualisation of transients in their target, the sarcomere, where calcium regulates contraction. This tool improves on established chemical dyes and untargeted genetically encoded indicators for analysing modulators of contractility and myofilament-based calcium signalling.

Calcium (Ca^{++}) is the only physiologically occurring ion that activates contraction[1]. This begins with voltage-dependent entry of extracellular Ca^{++} and is amplified by massive intracellular Ca^{++} release, principally from the sarcoplasmic reticulum (SR). The released Ca^{++} binds to Troponin C (TnC), promoting rearrangement of the thin filament allowing the rigid actin scaffold to interact with the motor protein myosin enabling ATP dependent contraction[2]. Relaxation occurs as Ca^{++} is removed from the myofilament and repackaged into the SR, or pumped out of the cell, for subsequent cycles.

Protein architecture within the sarcomere is highly ordered and densely packed, producing diffraction based striation pattern in unstained cells. Sarcomeric proteins are subject to point mutations and truncations in the common inherited heart diseases hypertrophic (HCM) and dilated (DCM) cardiomyopathy that change myofilament Ca^{++} sensitivity and/or contractility[3]. Modulators of contractility therefore represent potential therapies for these conditions, as well as the growing problem of heart failure[4] in general. Importantly they should act without changes to total [Ca^{++}] which is potentially pro-arrhythmic[5]. Therefore, to understand these diseases and identify such compounds, a need for myofilament [Ca^{++}] and contractility assessment exists where ideally measurement of one parameter does not alter the other.

Calcium handling studies in cardiomyocytes often use chemical dyes such as fura2[6], or colour variants based on the non-fluorescent Ca^{++} binder BAPTA. In a photometric system, designed to minimise dye use and light exposure, simultaneous fluorescence measurements of [Ca^{++}], by fura2, and contractility, by sarcomere shortening, in isolated ventricular adult guinea pig cardiomyocytes (vGPCMs) shows a progressive inhibition of contractility across the dose range used in imaging studies (**Supplementary Figure 1**). Although fura2-based impairment of contractility has been observed previously in isolated rat cardiac trabeculae[7], it is unclear if this can be entirely attributed to the sequestration of Ca^{++} by the typical 0.1-

40mM range that dyes accumulate at within cells[8,9] as effects on whole-cell contractility are observed with a chemical indicator of voltage, fluovolt[10], and the sodium indicator SBFI[11] (**Supplementary Figure 2**). These dyes do not bind Ca^{++} but do require weak surfactant application with or without acetoxymethyl esterification, to increase membrane permeability.

Recently, direct inhibition of the Na/K ATPase in many cell types by the BAPTA family of Ca^{++} indicators has been reported[12] raising the possibility that other ATPases may also be vulnerable to these chemicals. Indeed it appears that in vitro calcium regulation of the actomyosin ATPase is perturbed by fura2: in addition to reduced Ca^{++} sensitivity (due to calcium sequestration by the dye), the maximum rate of ATP hydrolysis is also depressed suggesting direct inhibition of the actomyosin ATPase itself (**Supplementary Figure 3**). Therefore, mechanisms beyond simple Ca^{++} buffering appear to cause contractile impairment. Dose response inhibition of contraction is also observed using iPS-derived cardiomyocytes (iPS-CM) (**Supplementary Figure 4**), limiting the utility of motion tracking contractility assessments[13] with simultaneous fura2 Ca^{++} imaging. Furthermore, since contractile suppression is detected during observer-independent impedance assessment in the presence of green-shifted calcium indicators on monolayer iPS-CM's from a different vendor (**Supplementary Figure 5**); UV light phototoxicity, cell singularisation, particular dyes or cell suppliers may be less important than the direct biochemical effects of these chemicals.

Genetically encoded calcium indicators (GECIs) offer an alternative strategy for [Ca^{++}] assessment[14], with the possibility of subcellular targeting and lower (~μM) expression levels. Myofilament specific probes however remained elusive, in part due to the belief that the bulky 30x40x70Å adducts of the smallest GECIs[15] may be poorly tolerated by the paracrystaline sarcomeric environment[3,16] leading to perturbed function of the indicator, the fusion partner, or the cell. We have developed a thin filament-restricted probe by screening green fluorescent protein (GFP) fusions with troponin subunits to identify sites that preserve

actomyosin ATPase regulation (**Supplementary Figure 6, Supplementary Table 1**). We find that GFP or the red calcium indicator RGECO[17], previously applied to vGPCMs and iPS-CMs[18], can be conjugated to the N-terminus of Troponin-T without disrupting in vitro thin filament reconstitution or the Ca^{++} sensitivity of the actomyosin ATPase (**Supplementary Figure 7**). In vitro characterisation of the calcium indicator properties of RGECO-TnT showed a K_d of 764.5±17.6 nM (RGECO was 860.0±10.0 nM) under physiological pH, temperature and magnesium concentration (**Supplementary Figure 8**), with preservation of dynamic range and excitation/emission spectra (**Supplementary Figure 9, Supplementary Table 2**). At physiological temperature, Ca^{++} on (at 10 μM free Ca^{++} k_{obs} 16.7 $\mu M^{-1}s^{-1}$) and off (k_{off} 16.9 s^{-1}) rates for RGECO-TnT were similar to RGECO (**Supplementary Figure 10**), and approximately twice those observed at 25ºC.

When RGECO-TnT is adenovirally expressed in vGPCMs (**Figure 1a**) or iPS-CMs (**Figure 1b**), in contrast to an unrestricted RGECO[18], sarcomeric localisation without M line or Z-disc accumulation is observed, and RGECO-TnT is incorporated at levels similar to endogenous TnT (**Figure 1c**). Unlike fura2, which relaxes the resting sarcomere length and reduces sarcomeric shortening, the contractile parameters in vGPCMs (**Figure 1d & e**) or iPS-CMs (**Supplementary Figure 4**) appear unaffected in the presence of either GECI. Ca^{++} transients are readily detected in electrically paced vGPCMs (**Figure 1f)** and iPS-CMs (**Figure 1g**), with reverse rate dependence. Dynamic range compression is apparent at higher pacing frequencies in vGPCM (**Supplementary Figure 11**) and iPS-CM (**Supplementary Figure 12**) as expected[18,19]. Spontaneous transients are also observed in iPS-CM (**Supplementary movie 1**). GECIs can be employed in combination with other indicators[14,17] or optical control tools[15,18]; indeed RGECO-TnT can be used simultaneously with the cytoplasmic green Ca^{++} indicator, GGECO[17] in iPS-CM's (**Supplementary Figure 13**).

There is interest in the therapeutic use of small molecule modulators of myofilament contractility[3,5,20,21]. Importantly these should not affect Ca^{++} handling which may be pro-arrhythmic. To contrast the sensitivities of RGECO-TnT, RGECO and fura2 and the mechanistic insights given by each, we investigated the effects of three drugs on Ca^{++} handling and contractility: the myosin ATPase inhibitor MYK-461[20], levosimendan[21] (a myofilament activator that stabilises the Ca^{++} bound form of TnC) and flecainide[18], which does not target the myofilament but has complex effects on sodium (SCN5a), and potassium (hERG) channels, and the ryanodine receptor (RyR2) (**Figure 2, Supplementary Tables 3a, 4a, 5a**).

Myofilament targeting appears to enhance reporter sensitivity to the underlying biology. For example, RGECO-TnT detects larger peak amplitude reductions or increases compared to the other indicators strategies when small molecules that act via the myofilament (MYK-461 and levosimendan) are used. This may result from subcellular proximity of the sensor to site of drug action. The effect of MYK-461 on Ca^{++} handling was unexpected. Previous reports using fura2 measurements in isolated rat cardiomyocytes showed no effect on Ca^{++} transients with similar drug concentrations[19]. Although one explanation for this is the choice of indicator, other differences like duration of fura2 exposure, data processing, or different electrophysiology and Ca^{++} handling of rat cardiomyocytes compared to guinea pig cannot be excluded at this point. When intracellular Ca^{++} was remotely modulated with flecainide, the unrestricted GECI sees no change in signal amplitude, in contrast the myofilament restricted GECI sees it fall. Together these data suggest that RGECO-TnT improves sensitivity to detect Ca^{++} handling at the level of the myofilament when compared to fura2 or an unrestricted RGECO. These treatments also illustrate how the RGECO-TnT adds mechanistic information when assessing intracellular Ca^{++} dynamics. Whilst all three sensors detect changes to T_{50} off times, neither fura2 or unrestricted RGECO reported

changes to T_{50} on rates. However, the myofilament restricted sensor appears able to identify these subtle changes.

Both GECI's offer significant advances during combined assessments of contractility (**Supplementary Figure 14, Supplementary Tables 3b, 4b, 5b).** While the presence of GECIs had no significant effect on sarcomere shortening, fura2 loaded cells relax and contract less and composite effects due to the action of both fura2 and drug are observed. For example, in indicator-free control vGPCM's, 250nM MYK-461 increases resting sarcomere length by ~0.1µm. Either GECI strategy identifies this, but paradoxically the resting length shortens in cells also loaded with fura2. Similarly, although levosimendan treatment increases the resting diastolic sarcomere length in control cells, in the presence of fura2 it apparently decreases. These observations reveal the vulnerability for synergistic, or opposing, actions of the chemical dye on contractility to accentuate, or ameliorate, phenotypes induced by drugs in single cells.

In summary, we have identified a site within the contractile machinery that tolerates delivery of genetically encoded reporters into the heart of the myofilament without compromising the function of the cell, the indicator, or the fusion partner. This allows detection of the [Ca^{++}] in the vicinity of the myofilament and permits simultaneous contractility measurements unaffected by the presence of the sensor, thus representing an improvement on the chemical dyes widely used in this field. This approach should also allow the targeting of other sensors to the myofilament to detect changes during development or disease.

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Methods: full methods are supplied in an online supplement

Funding:

Work in MJD's lab is supported by the Wellcome Trust (WT098519MA), YFC was supported by a pump priming grant from the BHF Centre of Excellence award to Oxford University (RE/08/0**04/**23915 & RE/13/1/30181). MJD's Cross appointment in Osaka is made possible by the JSPS International Joint Research Promotion Program to Osaka University. XZ & YAA are employees of ACEA Biosciences. PR, AS, HW and CR are

supported by the British Heart Foundation (Programme grant RG/12/16/29939) and the British Heart Foundation Centre of Research Excellence (Oxford).

Competing Financial Interests statement:

MJD, PR, AJS, CNB, KS, YFC and CR have applied for a patent on materials and methods related to the fusion proteins targeting the sarcomere (filing reference **TBC**)

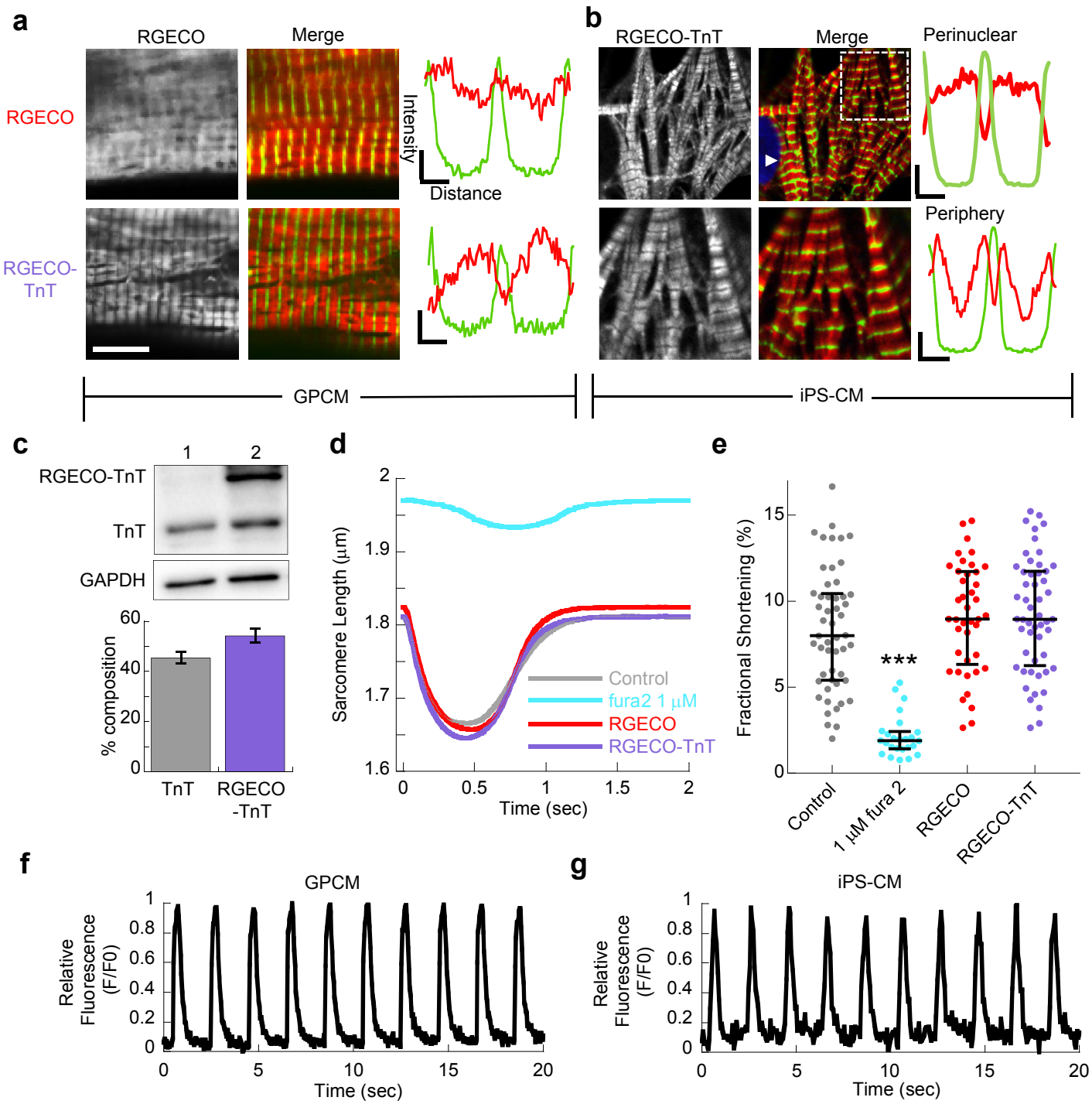

Figure 1. The red genetically encoded Ca^{++} sensor RGECO can be delivered to the myofilament by Troponin-T fusion revealing a dynamic signal from the sarcomere of adult ventricular and iPS derived cardiomyocytes without affecting contractility. In contrast to the diffuse staining of RGECO, adenovirally expressed RGECO-TnT localises to the I band in confocal microscopy of fixed adult ventricular **(a)** and human iPS derived cardiomyocytes **(b)**; z disks are revealed by α-actinin staining (green in the merge image). Scale bar = 10µm. Dashed box shows the region expanded in the zoom panel. Intensity profile plots spanning two sarcomeres (three z-discs, ~3.6µm) are shown adjacent to the merge image, α-actinin (green line) labels the z-disc, the Ca^{++} indicator (red) is excluded from the Z-disc by TnT fusion. Different sarcomere morphologies are present in iPS-CM, examples adjacent to the nucleus (white arrow head), or the periphery (zoom panel) are shown. Western blot analysis of vGPCMs expressing RGECO-TnT **(c)**, indicates expression equivalent to endogenous TnT (54.3±5.5%, n=5). Unloaded sarcomere shortening during electrical paced (0.5 Hz) of isolated adult cardiomyocytes was used to test cardiac contractile function in vGPCM's by measurement of the sarcomeric length during contraction **(d)** or by fractional shortening assessment **(e)**. RGECO (Red) and RGECO-TnT (Purple) infected cells were not significantly different to control (Grey), however, cells loaded with 1µM fura2 (Blue) displayed significant increase in resting sarcomere length, and blunted contractility in either assessment **(e)**. The trace recordings in **(f)** and **(g)** show the dynamic red fluorescence Ca^{++}signal acquired from RGECO-TnT expressing single GPCMs and iPS-CMs imaged at video rate during electrical pacing (0.5 Hz).

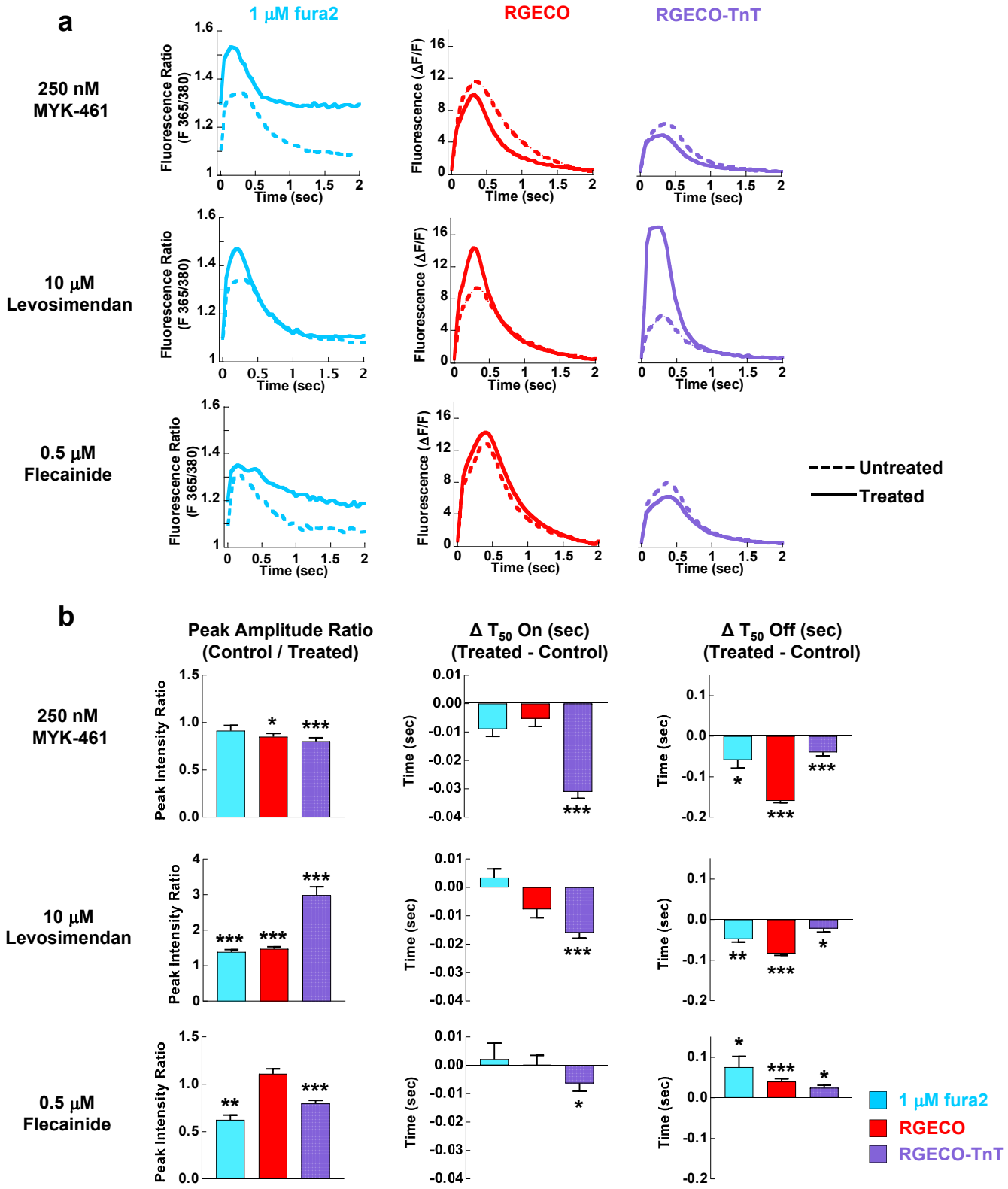

Figure 2. Small molecule effects on paired Ca^{++} transient measurements in vGPCM's are different when fura2 is used compared to a genetically encoded probe RGECO or RGECO-TnT. The effects of MYK-461 (a myosin-ATPase inhibitor that reduces myofilament Ca^{++} sensitivity), Levosimendan (a myofilament Ca^{++} sensitizer) and Flecainide (a sodium channel inhibitor with off target effects on the potassium channel hERG, and the Ryanodine receptor RyR2) on the Ca^{++} transient and contractility were explored in electrically paced (0.5 Hz) adult ventricular cardiomyocytes. The Ca^{++} dependent dynamic signals of 1 μM fura2 ($F_{365/380}$), RGECO and RGECO-TnT are shown in **(a)**. Each comparison is made in paired experiments between drug treated (solid lines) and DMSO control treated (dashed lines) for fura2 loaded (blue), RGECO infected (red) and RGECO-TnT infected (purple) cells. Δ values for all extracted parameters (including SEM and p values) for MYK-461, Levosimendan and Flecainide are plotted in **(b)**. Error bars are SEM, * = $p<0.05$, **= $p<0.01$ and *** = $p<0.001$ using students t-test comparing untreated to treated cells. Absolute numbers for extracted parameters for MYK-461, Levosimendan and Flecainide treatment are tabulated in **Supplementary Tables 3, 4 and 5** respectively.

METHODS

Primary Cardiomyocyte isolation

This investigation was approved by the Animal Welfare and Ethical Review Board at the University of Oxford and conforms to the UK Animals (Scientific Procedures) Act, 1986, incorporating Directive 2010/63/EU of the European Parliament.

Adenoviral Virus design and production

Recombinant adenoviral constructs were created using the AdEasy XL recombination system (Agilent technologies) as previously described[22]. Briefly; RGECO and RGECO-TnT (**Supplementary Figure 17)** (sub-cloned using a BamH1 linker), were inserted into a shuttle vector, containing a CMV promoter 3' of the multiple cloning site. The shuttle vectors were recombined in BJ5183-AD1 *E.coli* pre-transformed with the AdEasy viral backbone plasmid. Colonies positive for homologous recombination were confirmed with PacI and BstxI digest patterns. Successfully cloned AdEasy plasmids were linearized and transfected into an 80% confluent 25 cm^2 tissue culture flask containing (E1 a/b) modified (AD) HEK 293 cells that package the adenoviral particles for 3-4 weeks. Scale up of high titre adenovirus was achieved by sequential rounds of re-infection from cell lysates using increasing cell numbers to a final infection of fifty 175 cm^2 flasks of 90% confluent AD293 cells. The viral particles were purified using overnight CsCl gradient centrifugation and desalted in to a buffer containing 20 mM tris-HCl pH 7.0 and 7.5% sucrose. Desalted virus was mixed 1:1 with a solution containing 20% glycerol 80% FBS for cryo-protection and stored at -80 °C. Viral titre was estimated by infection of serial dilutions of purified virus in a plate containing ~$2x10^6$ unmodified HEK293 cells over a 48 hour period. Cells were fixed using in 4% PFA and chemically skinned using 0.1% Triton X-100. Permeablized cells were incubated sequentially with a 1 in 200 dilution of Ds-Red primary antibody (Living Colors, Takara Bio,

USA) and a 1 in 1000 dilution of Alexa 568 secondary antibody (Life Technologies, CA, USA). Subsequent cell counts of red fluorescent vs. total cell number gave a viable virus particle estimate of $5x10^{10}$/ml and $1.49x10^{9}$/ml for RGECO and RGECO-TnT respectively.

Isolation of guinea pig left ventricular cardiomyocytes

A 400 g male guinea pig (Harlan, Bicester, UK) was dispatched by cervical dislocation. The chest cavity opened, an 18 gauge cannula inserted into the aorta and fixed in place with a short length of sterile suture. The heart was excised and immediately hung on Langendorff apparatus which was water jacketed to 37 °C and in which all solutions were pre-oxygenated. The heart was perfused with isolation solution (130 mM NaCl, 23 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 21 mM glucose, 20 mM taurine, 5 mM creatine, 5 mM $MgCl_2$, 5 mM Na pyruvate, 4.5 mM KCl, 1 mM NaH_2PO_3, pH 7.3 with NaOH) containing 0.016 mM EGTA for 5 minutes. The heart was subsequently perfused with isolation solution containing 0.15 mM $CaCl_2$, 0.1% BSA, 0.8 mg/ml collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 0.06 mg/ml of protease from *Streptomyces griseus* for 5 minutes, followed by perfusion with isolation solution containing 0.15 mM $CaCl_2$, 0.1% BSA for 5 minutes to inactivate the collagenase. The left ventricle was cut away, mechanically homogenised and placed in 50 ml of isolation solution containing 0.15 mM $CaCl_2$, 0.1% BSA in a shaker at 37 °C for 10-15 minutes until a significant proportion of the excised tissue had broken up. Isolated cells were passed through a sterile gauze filter to remove any undigested tissue and centrifuged for 2 minutes at >100x*g*, the supernatant was removed and the cell pellet was resuspended in 50 ml isolation solution containing 0.5 mM $CaCl_2$ and immediately centrifuged for 2 minutes at >100x*g* , the supernatant was removed, the cell pellet was resuspended in 50 ml of isolation solution containing 1 mM $CaCl_2$ and immediately centrifuged for 2 minutes at >100x*g* and the

supernatant was removed. Finally, the cells were resuspended and plated at ~$1x10^5$ per ml in ACCITT3 cardiomyocyte culture medium as first described by Ellingsen et al[23] containing 500 ml M199 media 100 units of penicillin, 0.1 mg/ml streptomycin, 20 mM taurine, 5 mM creatine, 5 mM Na pyruvate, 2 mg/ml BSA, 2 mM L-carnitine, 0.1 pM insulin. Recombinant adenovirus was immediately added to 0.75 ml of cell suspension to an estimated multiplicity of infection (MOI) of between 500 and 800, infected cells were placed at 37 °C in a 5% CO_2 atmosphere for 48 hours. This was sufficient to infect 100% of all viable rod shaped cardiomyocytes with either RGECO or RGECO-TnT.

Stem cell derived cardiomyocytes

Human iPS derived cardiomyocytes and maintenance media were purchased from Axol Bioscience, (Cambridge, UK), and handled according to manufacturer's recommendations at 37 ºC, 5% CO2 in a humidified incubator. After 72 hours in culture they were singularised and re-plated onto Fibronectin (0.5%)/Gelatin (0.1%) coated 35mm glass bottom dish (No. 0) MatTek plates (Ashland, MA, US) at 1:6.

Protein purification

N and C terminal eGFP conjugates of human recombinant TnT and TnC were produced by molecular cloning in PMW172 vector using flanking 3' NdeI and 5' EcoRI restriction sites and an internal HindIII restriction site used as a linker in each case (NEB, New England). RGECO and RGECO-TnT were also sub-cloned in to PMW172 using the same strategy. Proteins were expressed in BL21-DE3-pLysS *E.coli* induced using 0.4 mM Isopropyl β-D-1-thiogalactopyranoside for 4 hours. Bacterial pellets were recovered by centrifugation (10,000 x*g* for 10 minutes) and lysed in a buffer containing 25 mM Tris HCl, pH 7.5, 20% sucrose, 1 mM EDTA, 200 mM NaCl, 5 M urea, 0.1% Triton X-100. All proteins were purified using an AKTA-UPC900 FPLC using HiTrap FF chromatography columns (GE Healthcare,

Amersham). GFP-TnT and TnT-GFP were purified using sequential cation (in buffer containing 6 M urea, 1 mM EDTA, 1 mM 2-mercaptoethanol, 20 mM MOPS, pH 6.0) followed by anion (in buffer containing 6 M urea, 1 mM EDTA, 1 mM 2-mercaptoethanol 50 mM Tris HCl pH 8.0) exchange chromatography. GFP-TnC and TnC-GFP were purified by anion (pH 8.5) exchange chromatography. RGECO and RGECO-TnT were purified using sequential anion (pH 8.0) exchange chromatography, Ammonium Sulphate fractionation (to 50% for RGECO and 35% for RGECO-TnT) and finally Hydrophobic Interaction Chromatography (HIC) (in buffer containing 30% ammonium sulphate, 200 mM $NaCl_2$ 1 mM ditiothretol and 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)). Ion exchange columns were eluted using a gradient of 0-2 M NaCl, the HIC column was eluted with a gradient of 30–0% ammonium sulphate. All eluted protein fractions were assessed for purity using 12% SDS PAGE gels, stained with coomassie brilliant blue. Wild type human recombinant TnT, TnI, TnC and Ala-Ser-α-TM[24] were purified as previously described[25]. Troponin complex containing either WT, GFP-TnT, GFP-TnT, GFP-TnC, TnC-GFP or RGECO-TnT were reconstituted by dialysis into buffer containing 10 mM imidazole pH 7.0, 1 mM DTT, 0.01% azide, 0.1 mM $CaCl_2$, 6 M urea and 1 M KCl, firstly urea was reduced stepwise from to 2 then 0 M then KCl was reduced stepwise from 1M to 800 – 600 – 400 - 200 mM KCl in a series of 3 hour dialyses. Tn Complex was purified using size exclusion chromatography in 200 mM KCl dialysis buffer (**Supplementary Figure 8b**), purity was analysed by SDS PAGE (**Supplementary Figure 8c**), and finally, purified troponin complexes are dialysed into buffer containing 5 mM 1,4-Piperazinediethanesulphate pH 7.0, 3,87 mM $MgCl_2$, 1 mM DTT for ATPase assay experiments. Actin and myosin-S1 was extracted from rabbit skeletal muscle as previously described[26,27].

Actin Co-sedimentation assays

Reconstituted Tn complex proteins (0.5 μM) were co-sedimented with human Ala-Ser-α-TM (0.5 μM) and rabbit skeletal actin (3.5 μM) at 384000 x*g* for 15 minutes by ultracentrifugation (Beckman Coulter Inc, High Wycombe). Total, supernatant and pellet fractions were visualized using coomassie blue stained 12% SDS PAGE gels in order to ensure completed reconstitution of thin filaments prior to all ATPase assay measurements.

In vitro acto-myosin activated myosin ATPase assays

ATPase assays were undertaken using our standard assay conditions[25,28]. Briefly, a master stock of 3.5 μM actin, 0.5 μM myosin S1, 0.5 μM Ala-Ser-α-TM and 0.5 μM Tn complex were mixed in buffer containing 5 mM 1,4-Piperazinediethanesulphate pH 7.0, 3.87 mM $MgCl_2$, 1 mM DTT. In order to ensure precise thin filament protein stoichiometry, each stock was centrifuged at 384000 x*g* for 15 minutes. Supernatants were discarded, actin pellets were recovered in buffer containing 5 mM 1,4-Piperazinediethanesulphate pH 7.0, 3.87 mM $MgCl_2$, 1 mM DTT to an equal volume of the discarded supernatant. Reaction mixtures were aliquoted and set to a range of $_{free}[Ca^{++}]$ between 3.16 nM (pCa 8.5) and 31.6 μM (pCa 4.5) using 1 mM EGTA and the appropriate corresponding concentration of $CaCl_2$, calculated using Maxchelatior software (http://maxchelator.stanford.edu/CaEGTA-TS.htm). ATPase reactions were begun with the addition of 3mM ATP and incubated at 37°C for 15 minutes. Each reaction mixture was quenched in 500μl of trichloroacetic acid and the amount of inorganic phosphate released measured by the addition of 500μl of 1% ammonium molybdate in 0.5 M sulphuric acid, followed by 500μl of 40% iron (II) sulphate in 0.5 M sulphuric acid. Absorbance (A_{700}) measurements were converted to absolute activity (sec^{-1}) and calcium-sensitivity data was fitted to the Hill equation using Kaleidagraph (Synergy Software, Inc (USA)).

Hill equation: $$A = \frac{A_{\min} + (A_{\max} - A_{\min})}{1 + 10^{((pCa - pCa_{50}) \times n_H)}}$$

Where: A = ATPase rate; A_{min} = Minimum ATPase rate; A_{max} = Maximum ATPase rate; pCa = -log [Ca^{++}]; pCa_{50} = - log [Ca^{++}] required for half maximum ATPase activity; n_H = Hill coefficient.

Calcium binding, K_d and kinetic calculations

To measure steady state Ca^{++} binding affinity (K_d) for RGECO and RGECO-TnT, 3µM of protein was dialysed into buffer containing 130mM NaCl, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 1mM dithiothretol , pH 7.3 and either 0, 1.3 or 3.9 mM $MgCl_2$. A range of $_{free}[Ca^{++}]$ conditions were set between 3.16nM (pCa 8.5) and 31.6µM (pCa 4.5) using 1mM EGTA and the appropriate corresponding concentration of $CaCl_2$. Steady state fluorescence readings were made in an Ultraclear bottom 96 well microplate using a FLUOstar Omega plate reader (BMG LABTEC, Ortenberg, Germany), using a 544nm excitation filter and 590/10nm emission filter at both 25 and 37°C. Resultant fluorescence emission intensities were plotted vs $_{free}[Ca^{++}]$ and fitted to the Hill equation to calculate K_d values for each protein and condition.

To measure Ca^{++} displacement (k_{off}), 125nM RGECO or RGECO-TnT plus 5µM $CaCl_2$ was mixed with 5mM EGTA in a buffer containing 130mM NaCl, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 1.3mM $MgCl_2$, 1mM dithiothretol, pH7.3 with NaOH. RGECO or RGECO-TnT was loaded into a Stopped-flow system (HiTech Scientific, Bradford-on-Avon, UK), concentrations after mixing 1:1 in the stopped-flow. Fluorescence was excited at 546nm (100W Xe/Hg lamp and monochromator) and emission monitored through a OG-590 glass filter. The resultant data was fitted to a single exponential decrease in fluorescence of 50-100%, depending upon temperature. To measure calcium binding (k_{on}),

125nM RGECO or RGECO-TnT was measured at 10µM free calcium by mixing with a buffer containing 2.125mM Ca.EGTA and 0.2mM EGTA. The stopped-flow fluorescence filter set up was idetical to that of the displacement measurements. A large increase in fluorescence upon addtion of calcium was fitted to a single exponetial. The observed single exponential rate constant, k_{obs}, was extracted for k_{on} and k_{off} for both sensors, at a range of temperatures between 25 and 37°C. To estimate the dissociation constant we using the equations $K_d = k_{obs\text{-}off}$ [Ca]/ k_{obs} on. Which were of comparable values to steady state K_d values obtained previously.

Determination of Quantum Yield and Molar Extinction Co-efficient

Standards for quantum yield determination were mCherry (for RGECO) and RGECO for RGECO-TnT. Briefly, the concentration of protein in buffer containing 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 1.3mM $MgCl_2$ and 1mM dithiothreitol was determined using BCA assay (Pierce) and set to either pCa 4.5 of 8.5, the concentration of each protein was reduced to an absorbance at the excitation wavelength between 0.2 and 0.6. A series of dilutions to absorbance's of 0.01, 0.02, 0.03 and 0.04 were made for each protein solution and standard. The fluorescence emission spectra at Ex F_{568} of each dilution of each standard and protein solution were recorded using an RF-1501 spectrofluorimeter (Shimadzu, Kyoto, Japan). The total fluorescence intensities were then obtained by integration. Integrated fluorescence intensity vs. absorbance was plotted for each protein and each standard. Quantum yields were determined from the slopes (S) of each line using the equation: Φprotein = Φstandard × (Sprotein/Sstandard).

Extinction coefficients were determined by first measuring the absorption spectrum of both RGECO and RGECO-TnT at pCa 8.5 and 4.5 in the buffer above using a plate reader (BMG LABTECH, Offenburg, Germany). Peak Absorbance wavelengths were determined to be

A_{465} in low Ca^{++} and A_{581} in high Ca^{++}. Absorbance measurements were automatically converted to 1 cm path length using BMG Omega software. Extinction coefficients of each protein were calculated by dividing the peak absorbance maximum by the previously determined concentration of protein.

Chemicals

Research grade chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise specified.

Chemical dye preparation & loading

Fura-2, FluoVolt, SBFI-AM were purchased from Thermo Fisher. FluoVolt and SBFI were prepared in DMSO as per manufactures instructions. FLIPR Calcium 5 was obtained from Molecular Devices and used as per manufacturer's instructions.

Small molecules

MYK-461 was purchased from Cayman Chemicals, Levosimendan from Sigma, and Flecainide from Abcam. All were prepared in DMSO as per manufactures instructions, and used at final concentrations as specified.

Measurement of sarcomere shortening and fura2 Ca^{++} transients

Sarcomere shortening and Ca^{++} transient measurements were performed using IonOptix µstep apparatus and the manufacturers' standard operating instructions. Briefly: cultured cardiomyocytes were loaded with fura2 Ca^{++} indicator by incubation with either 0.1, 0.5, 1.0 or 5.0µM fura2-AM ester (Life Technologies) in the presence of 2µM F127 pluronic in 'perfusion buffer' (150mM NaCl, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 7mM glucose, 1mM MgCl , 1mM KCl, 0.3mM NaH_2PO_3, pH 7.4 with NaOH) containing 250µM $CaCl_2$ for 5 minutes, followed by a 10 minute wash in perfusion

buffer containing 500µM $CaCl_2$ to remove any excess label. For experiments using SBFI 0.1, 0.5, 1.0 or 5.0 µM were loaded in the same manner. Experiments using FluoVolt (ThermoFisher) a 1/1000 dilution was used according the manufacturer's instructions. The loaded cells were then allowed to settle to the bottom of a perfusion chamber with a 1.5 thickness cover slip base, which was mounted on an inverted fluorescence microscope. Cells were perfused with Perfusion buffer containing 1.8mM $CaCl_2$, and electrically paced at 40 volts. Pacing frequency was set at 0.5 Hz in order to accurately measure resting diastolic $[Ca^{++}]_i$. Sarcomere shortening was captured by Fourier transform of the cardiomyocyte striations under phase contrast microscopy using a switching rate of 100 Hz. fura2 Ca^{++} transients were captured simultaneously, using the ratio of fura2 fluorescence emission at 365/380 nm at a switching rate of 1000 Hz. All contracting cardiomyocytes were measured for contractility and fura2 Ca^{++}, any cells displaying asynchronous contractility, excessive blebbing/dysmorphology were ignored for acquisition, whilst any cell with contractile magnitudes or velocities exceeding 2 standard deviations from the mean upon analysis were also excluded as product of phenotypic heterogeneity of primary cells in culture losing function and morphology over time. To compare sarcomere shortening and fura2 Ca^{++} transients in the presence of contractility or Ca^{++} modifying small molecules MYK-461 (0.25µM), Levosimendan (10µM) and Flecainide (0.5µM), 200 µl of cultured (48 Hours) cardiomyocytes were incubated in either drug or DMSO vehicle for 5 minutes. The entire cell preparation was pipetted onto the microscope perfusion chamber buffer containing the either drug or vehicle was perfused at 0.75 ml/min for the duration of the experiment. All contracting cells were measured (except those not meeting the exclusion criteria above), no preparation of cells was left for more than 15 minutes before being replaced for a fresh batch of pre-incubated cells. At least 3 cell preparations were analyzed for each DMSO/drug

comparison. No differences or clustering was observed between each cell preparation assessed.

RGECO imaging in fixed cells

Adult ventricular cardiomyocytes were exposed to recombinant adenovirus immediately after isolation in 0.75 ml of cell suspension to an estimated multiplicity of infection (MOI) of between 500 and 800, infected cells were placed at 37 °C in a 5% CO_2 atmosphere for 48 hours. At 48 hours an aliquot was taken and paraformaldehyde to a final concentration of 4% was added, at 10 minutes cells were pelleted (600rpm, 2min) in a benchtop centrifuge, washed with 1xPBS and resuspended in PBS. Cells were spun (Cytospin, Thermo Scientific) onto glass slides, and ringed with a PAP pen (Sigma). Human iPS-derived cardiomyocytes were plated as above and incubated to day 7 following thaw before washing with 1xPBS and fixation with 4% paraformaldehyde. For infection, virus was diluted 1:10 from the stock solution and used at 1 µl/dish 24 hours after replating. Permeabilisation with 0.1% Triton X-100 in Tris Buffered saline for 10 minutes at room temperature was followed by blocking (0.2% albumin in permeabilisation buffer) for 20 minutes. Primary antibodies (Mouse monoclonal anti-alpha actinin (Sigma, clone EA-53) rabbit polyclonal anti-DsRed (Clontech) were diluted 1:2000, and 1:200 in blocking buffer respectively. Three hours after primary incubation cells were washed in permeabilisation buffer, and counter stained with Alexa-488 anti-mouse, and Alexa-568 anti-rabbit fab fragment secondary antibodies (Invitrogen), nuclear counterstaining was with TO-PRO-3 (Invitrogen), for an hour before washing and mounting (Vectashield, Vector labs). Images were acquired on a Leica SP5 confocal microscope with a 63x oil immersion lens, and intensity plots prepared with the plot profile function in ImageJ.

Live cell Imaging Microscope

An inverted IX81 frame (Olympus, Japan), with a custom 7 LED array (Cairn Research, Faversham, UK), automated stage (Prior Scientific, Cambridge, UK), lens turret, and filter wheel is housed in a custom heated, humidified chamber (Solent Scientific, Segensworth, UK) with image collection on two C-1900 EMCCD cameras (Hamamatsu, Japan) mounted on a DC2 beam splitter (Photometrics, UK). For isolated RGECO imaging, single camera mode image acquisition through an Olympus LUCPlanFLN 40x objective lens (NA 0.60), or an Olympus UPlanFLN 10x lens (NA 0.3), using CellR software (Olympus) was used with uninterrupted recording of the calcium transient via a 5msec 568nm LED illumination pulse and the RFP filter set (DS/FF01-560/25-25, T565lpxr dichroic mirror, and ET620/60 emission filter, all Semrock). Simultaneous calcium transients from GGECO and RGECO indicators was acquired through an Olympus PlanApo 60x oil objective lens (NA 1.42), through a quad-band filter set (band pass filter (DS/FF01-387/485/559/649-25), dichroic quad-edge beam splitter (DS/FF410/504/582/669-Di01-25x36), and quad-band emission filter (DS/FF01-440/521/607/700-25) all Semrock with fluorescent emissions recorded through a Dual-View system (DC2, Photometrics) with green (520/30nm) and red (630/50nm) channels to separate cameras controlled through CellR (Olympus, Japan). RGECO and RGECO-TnT adenovirally induced vGPCMs were treated with 250nM MYK-461, 10µM Levosimendan, or 0.5µM Flecainide for 20 minutes then video rate movies with 0.5Hz pacing were acquired for 28 secs using the UPlanFLN 10x lens.

Transient analysis software & statistics

Raw image data was extracted using CellR (Olympus), and analysed in Excel (Microsoft). From a single cell 10 intensity transients over time were extracted and averaged to give a

single transient in a 2 second interval per cell; 3 transients were averaged for iPS-CMs. Time to 50% contraction, or time to 50% baseline (from peak) was determined from these traces.

Comparative peak intensity analysis was performed using paired control vs drug exposed datasets obtained from the same cell preparation. In brief the ΔF/F measurement described for intensiometric indicators[29] was used.

$$\frac{\Delta F}{F} = \frac{F(t) - F(min)}{F(min)}$$

Where $F(t)$ is the fluorescence intensity at time (t), and F(min) the minimum fluorescence intensity during the 2 second averaged dataset taken to be representative of a single cell.

Comparisons presented in Figure 2b were obtained by dividing or subtracting the relevant value of the paired control population from that of the drug exposed population based on the data in Supplementary tables 3, 4 & 5.

Where relative intensity data is presented, the following calculation was used

$$I = \frac{F(t) - F(min)}{F(max) - F(min)}$$

Where I, intensity, is a ratio of the fluorescence signal ($F(t)$) scaled according to the maximum ($F(max)$), and minimum ($F(min)$) response of the cell during the observation.

Unfiltered transient graphs were plotted using KaleidaGraph (Synergy Software), and dot plots with GraphPad Prism (GraphPad software). Groups were compared using unpaired Students t-test (MYK-461, Levosimendan, Flecainide) or one-way ANOVA with post-hoc Dunn's test (pacing frequency comparisons). Simultaneous RGECO and GGECO calcium traces from a single cell, and average responses at different pacing frequencies were normalized for presentation.

iPS-derived cardiomyocyte movement analysis

Cells plated and prepared as above were imaged in bright field mode at video rate over a 2 minute interval using the LUCPlanFLN 40x lens. Quantification of cell activity was scored for actively/non-actively contracting cells. An activity contracting cell was regarded as a cell presenting at least a single contraction within the 2 minute interval. The spontaneous beat frequency for these cells is 0.52 Hz +/- 0.15 Hz, with >90% cardiomyocyte purity based on TnI positivity[18].

Impedance assessment of contractility

iCell cardiomyocytes (CDI) were seeded at 20,000 cells/well into RTCA plates and loaded onto an xCELLigence RTCA Cardio system (ACEA bioscience) housed in a humidified incubator at 37 °C with a 5% CO_2 atmosphere, this was interrupted for feeding on alternate days. Contractility was monitored via impedance assessment at 12.9 msec intervals. At day 14 post seeding, with robust signals apparent in all wells, cells were loaded with FLIPR 5 (Molecular Devices) around the recommended 1x final working concentration. The recommended loading phase for FLIPR Calcium 5 is 1hr, recording continued for 24hrs. Raw transient data for the windows indicated together with transient amplitude and beat rate over the first two hours of the experiment were extracted using the RTCA software and prepared for presentation using Microsoft Excel.

Predictive structural modelling

The predicted models for the RGECO-TnT fusion were generated using RaptorX[30]using the primary amino acid sequence provided in **Supplementary figure 17**.

Supplementary References

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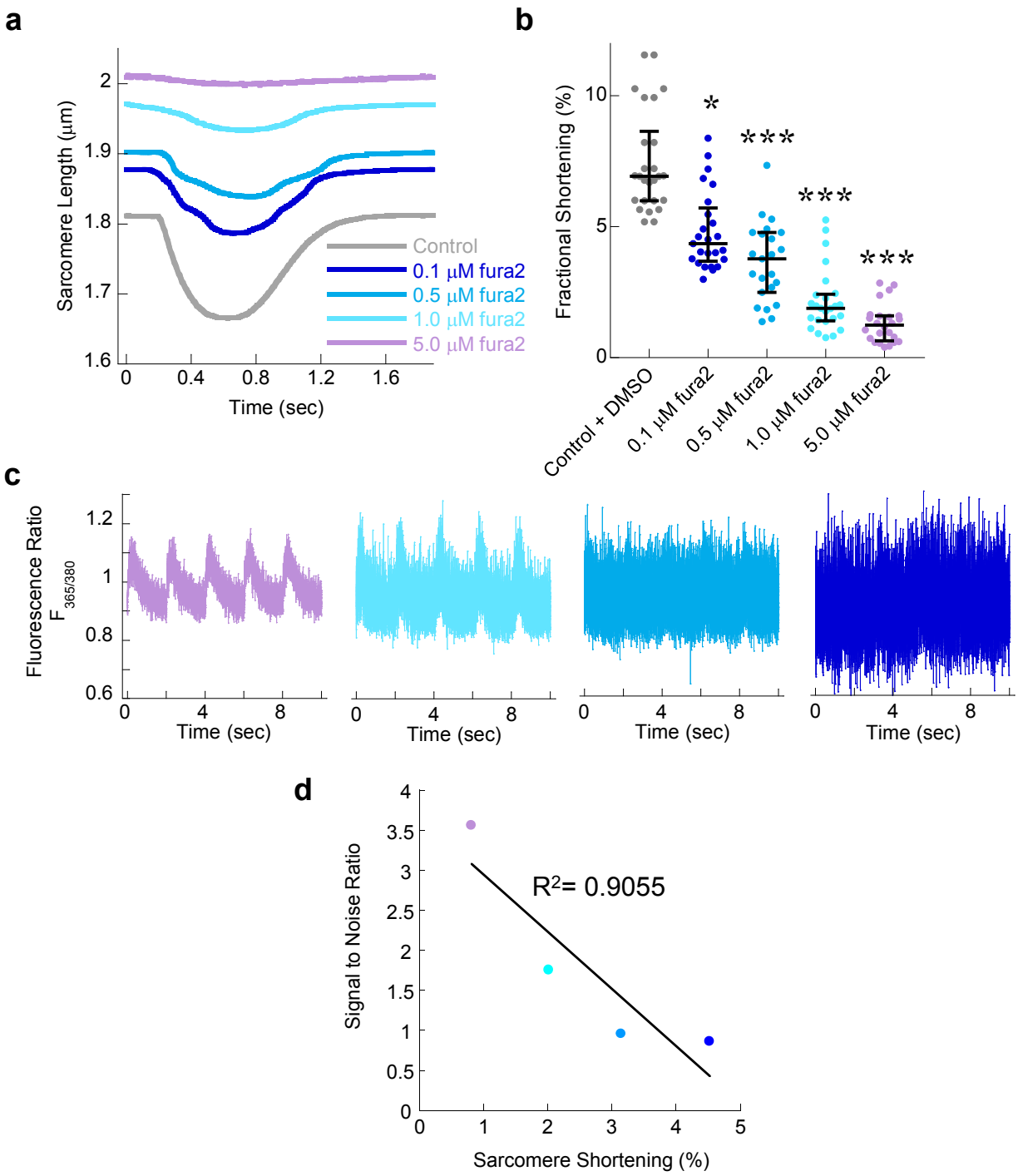
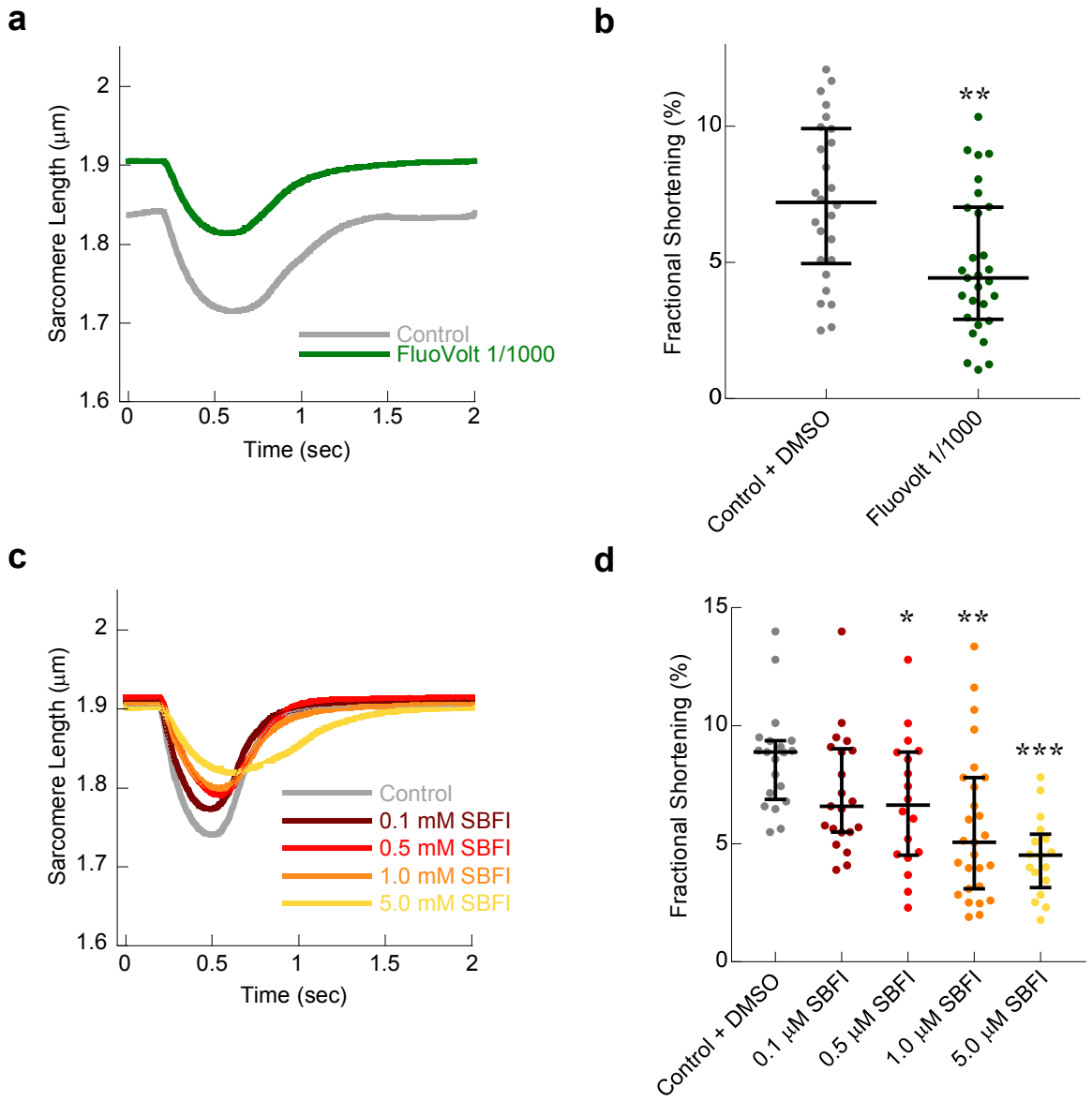
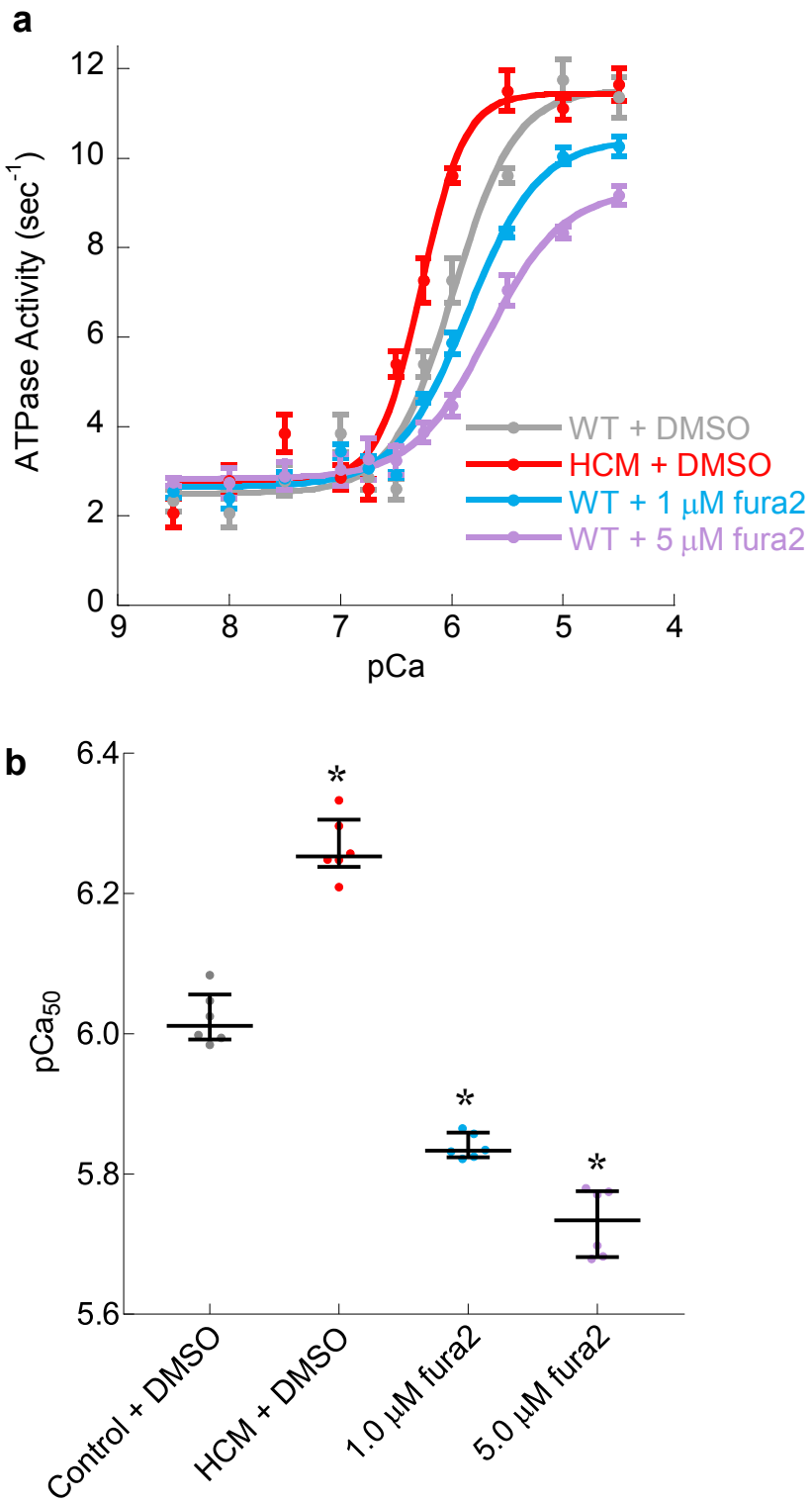


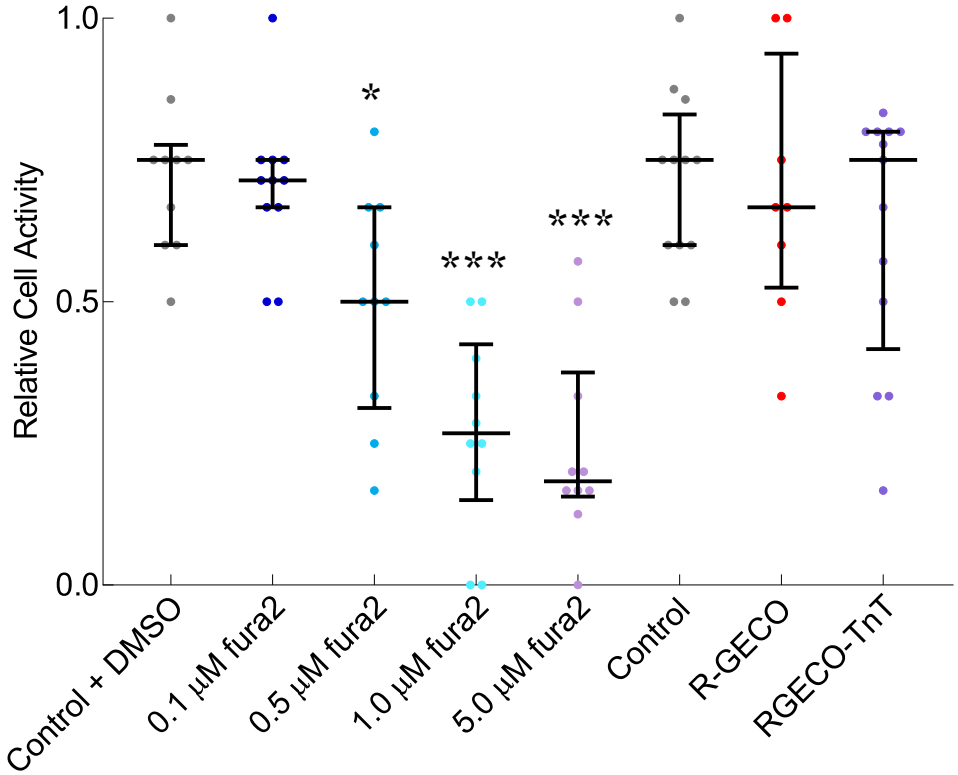
Supplementary Figure 1. fura2-AM ester reduces sarcomere shortening magnitude and speed in a dose dependent manner. Unloaded sarcomere shortening of electrically paced (0.5 Hz) isolated adult cardiomyocytes was used to test cardiac contractile function in **(a)**. Control (grey) was compared to cells loaded with 0.1 µM (royal blue), 0.5 µM (blue), 1.0 µM (teal) or 5.0 µM (mauve) fura2-AM ester. Significant reduction in fractional shortening was observed in each case **(b)** (lines give the median average and error bars are ± interquartile range * = $p<0.05$ and *** = $p<0.001$ using one way ANOVA). Example traces taken from pairwise acquisition of Ca^{++} dependent dynamic signal ($F_{365/380}$) in **(c)**, showed that it was necessary to load cardiomyocytes with >1 µM fura2 in order to observe a transient signal. A clear negatively correlated relationship exists between signal to noise of the Ca^{++} transient and fractional shortening of the sarcomere in vGPCM's is seen **(d)**. (n=23-26).



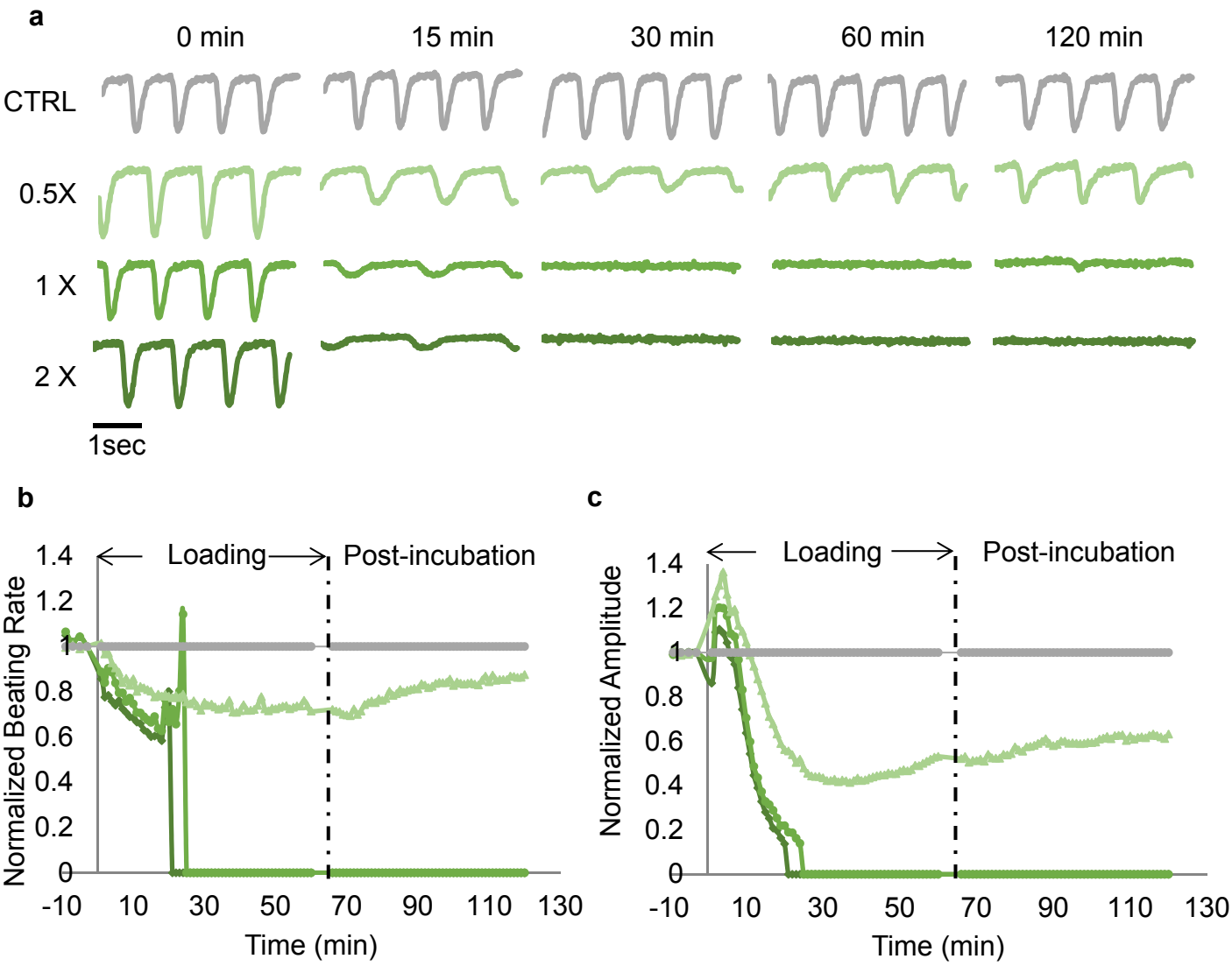
Supplementary Figure 2. Fluovolt-AM ester reduces sarcomere shortening magnitude and SBFI-AM ester reduces sarcomere shortening magnitude without affecting basal sarcomere length. Unloaded sarcomere shortening of electrically paced (0.5 Hz) isolated vGPCMs were used to compare control (grey) to cells loaded with 1/1000 dilution of Fluovolt-AM ester (green) loaded according to the manufacturers instructions **(a)**. Working concentrations of Fluovolt-AM ester reduced contractile magnitude and caused basal relaxation of cardiomyocytes **(b)**, however this effect was to a lesser extent than working concentration of fura2-AM ester (1 µM) determined in **Supplementary Figure 2**. **(c)** compares control (grey) to cells loaded with 0.1 µM (brown), 0.5 µM (red), 1.0 µM (orange) or 5.0 µM (yellow) SBFI-AM ester. Exposure to increasing concentrations of the sodium dye SBFI-AM ester also reduced contractile magnitude however did not affect basal relaxation of cardiomyocytes **(d)**. Lines give the median average and error bars are ± interquartile range, * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ using one way ANOVA



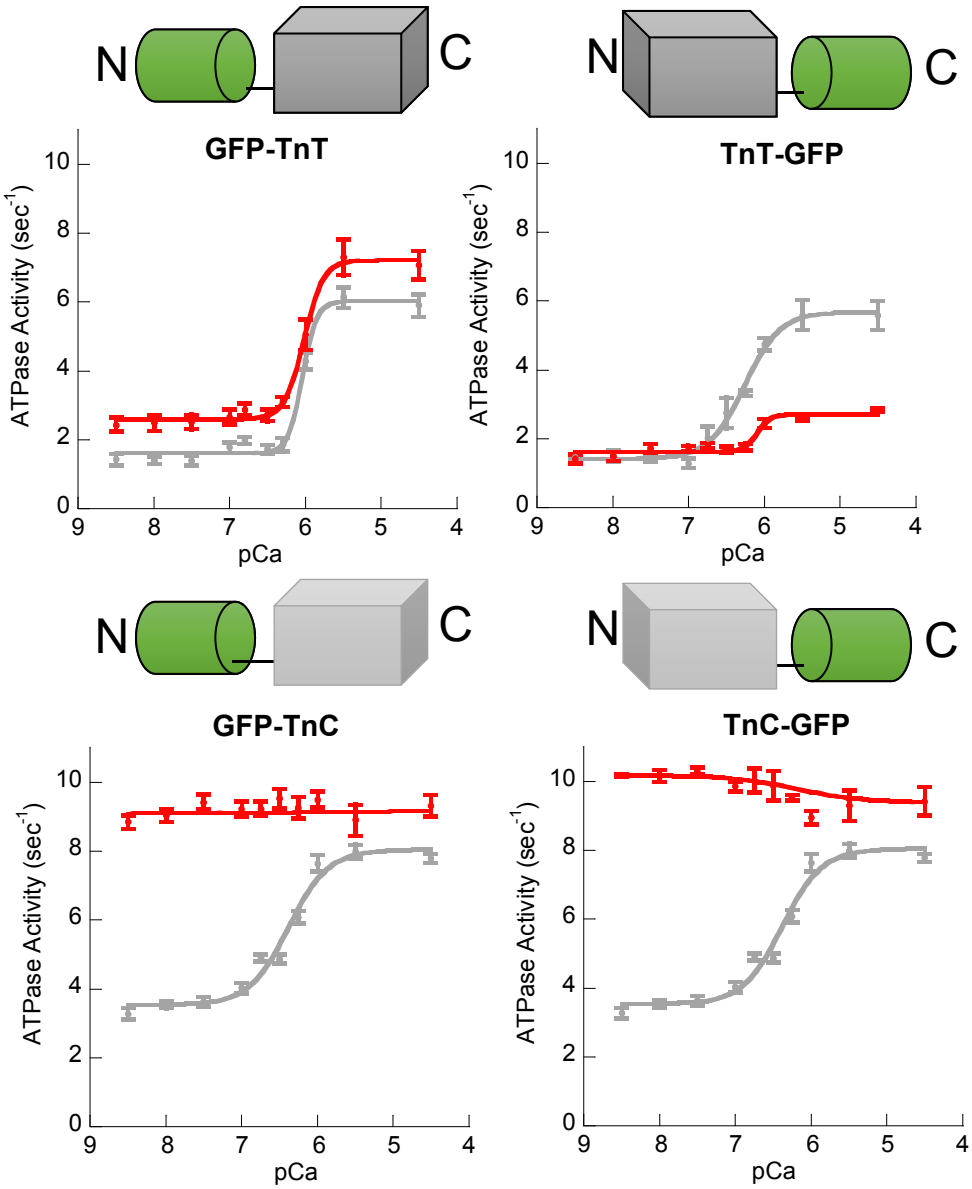
Supplementary Figure 3. fura2 reduces the Ca^{++} sensitivity of myofilament ATPase. Myofilament function was assessed using *in vitro* actin activated acto-myosin S1 ATPase assays. Myofilaments containing wild type control Tn complexes in DMSO (grey) were compared to troponin complex reconstituted HCM mutant (cTnT (R92Q) subunits in DMSO (red) or WT Tn with 1 μM (blue) or 5 μM (mauve) fura2-AM ester. Note the shift of the trace down, indicative of myosin ATPase inhibition, as well as right **(a)**. n= 5 error bars are ±SEM. Whilst HCM mutations in the myofilament increased Ca^{++} sensitivity (pCa_{50}) the presence of fura2-AM ester reduced pCa_{50} **(b).** These issues may confound the investigation of disease mechanisms in intact cellular assay systems. Lines give the median average and error bars are ± interquartile range, * = $p<0.05$ using one way ANOVA.



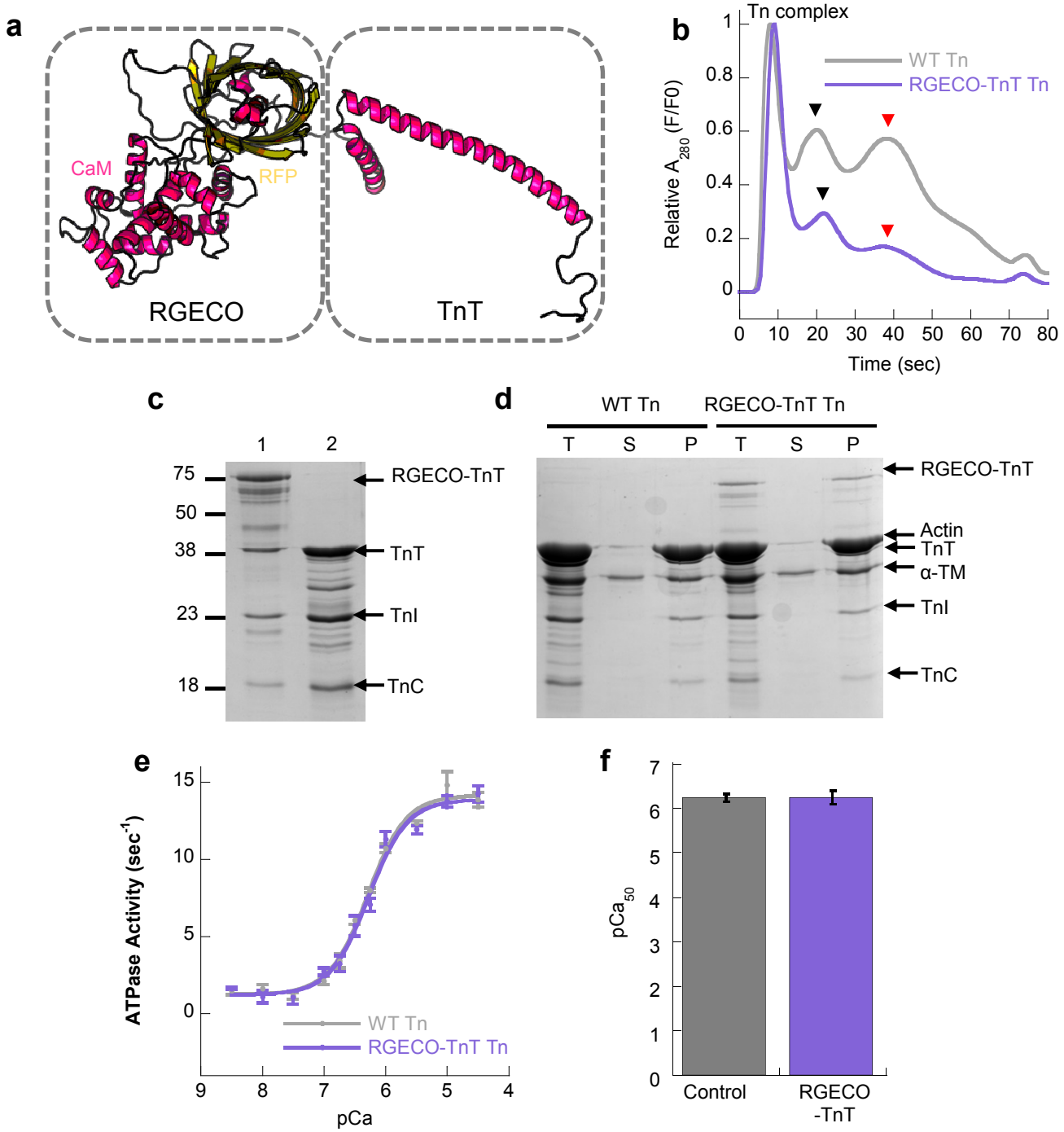
Supplementary Figure 4. fura2-AM ester reduces the relative contractile activity of human iPS-CMs whilst RGECO or RGECO-TnT do not. White light observation of singularised iPS-CMs was used to determine the fraction obviously contracting within a field of view over a two minute interval. Control (with and with out DMSO) (grey) were compered to cells loaded with 0.1 µM (royal blue), 0.5 µM (blue), 1.0 µM (teal) or 5.0 µM (mauve) fura2-AM ester or cells infected to express RGECO (red) or RGECO-TnT (purple) respectively. Significant reductions in contractile activity were observed in the presence of fura2-AM ester above concentrations of 0.5 µM. Lines give the median average and error bars are ± interquartile range * = $p<0.05$ and *** = $p<0.001$ using one way ANOVA.



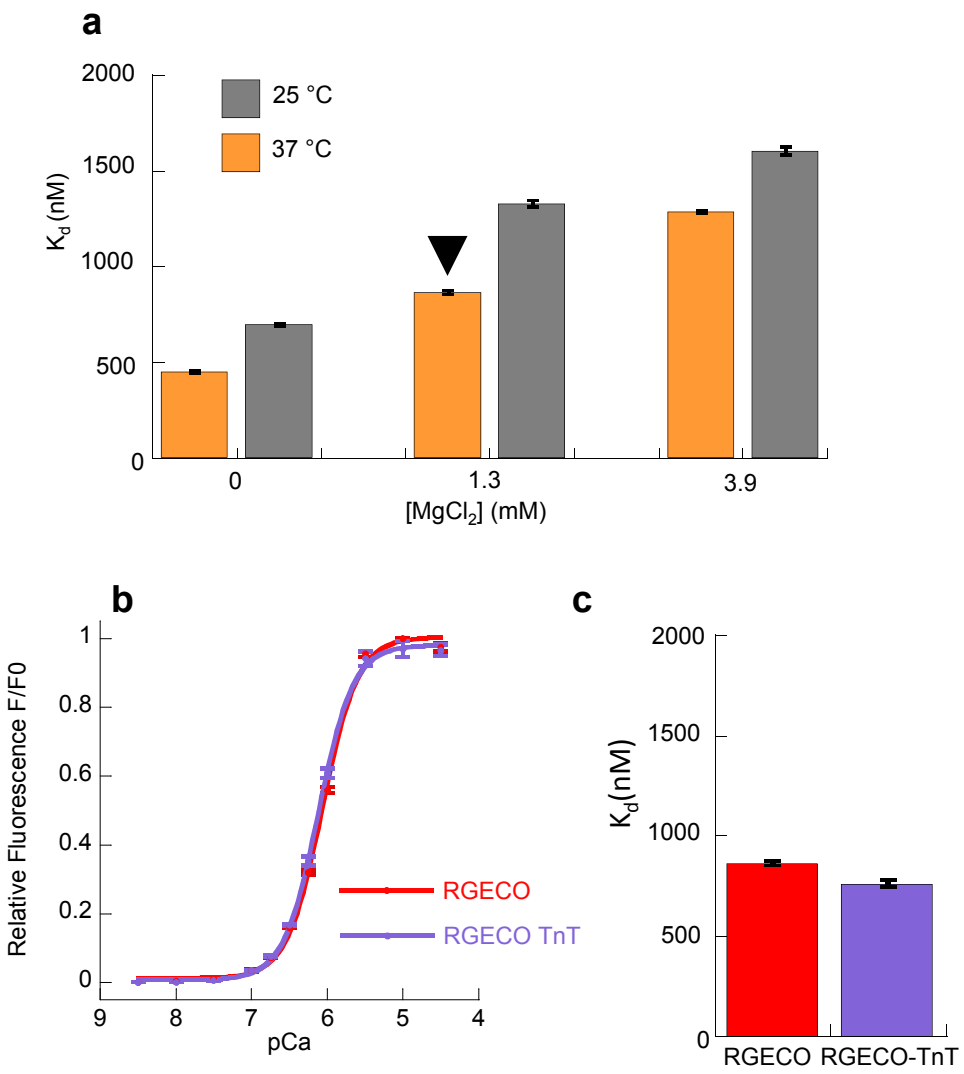
Supplementary Figure 5. The Ca^{++} dye FLIPR Calcium 5 reduces the contractile activity of human iPS-CMs. Label-free impedance monitoring to quantitatively evaluate iPSC-CM contractility in real-time, in the absence of light was undertaken with the Xcelligence RTCA Cardio system using 20 000 CDI cells in the presence of serial dilutions of the Ca^{++} dye FLIPR 5. Representative data windows of the impedance trace are shown for the one hour dye loading and subsequent one hour observation period (**a**). Dye incubation reduces the measured beat rate (**b**) and beat amplitude (**c**) before evidence of contractility is completely lost. Dye doses around the recommended final 1x concentration are shown as increasingly dark green lines in **a, b** & **c,** the vehicle treated control is in grey.



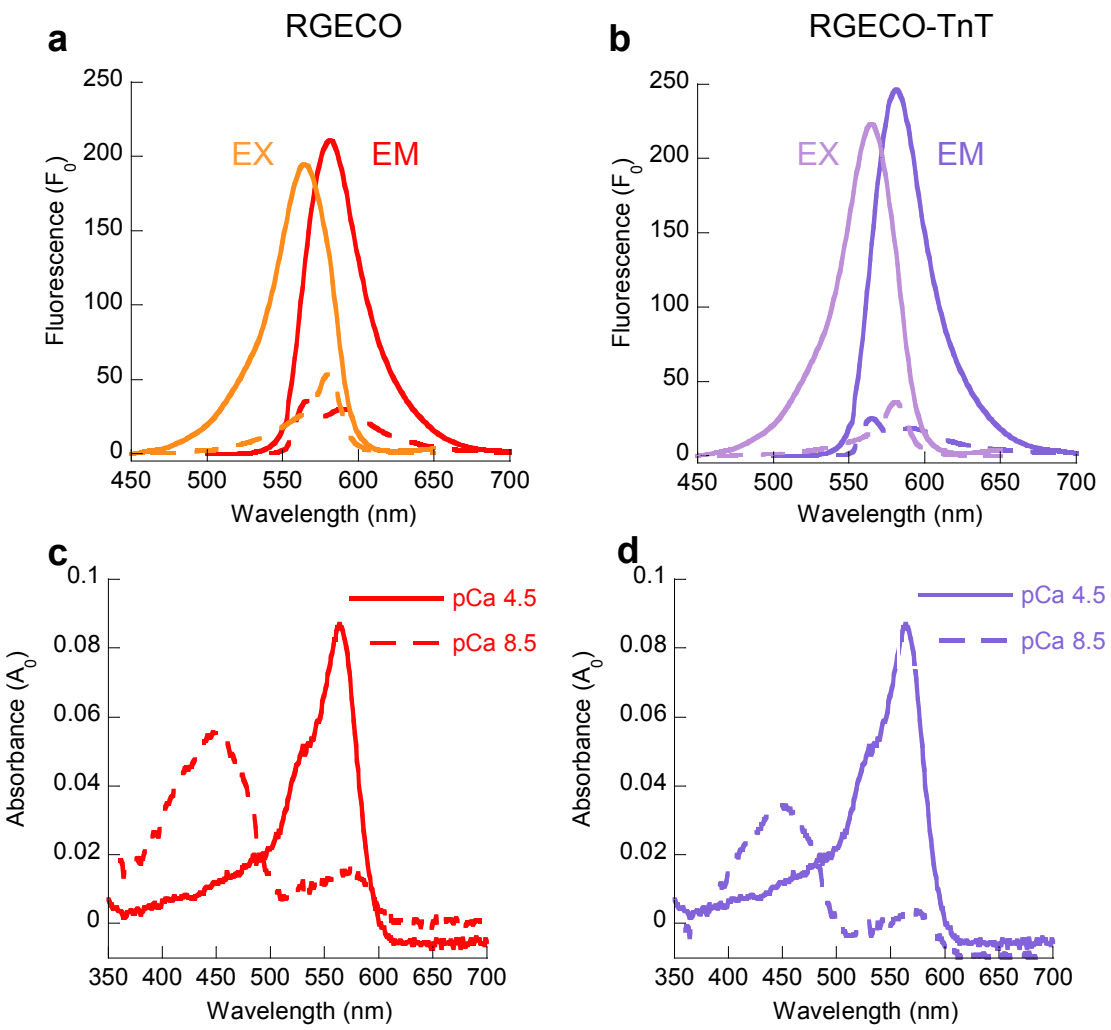
Supplementary Figure 6. The effect of GFP conjugation to the N and C terminus of troponin complex proteins on myofilament function. Myofilament function was assessed using *in vitro* actin activated acto-myosin S1 ATPase assays. Control (unconjugated) troponin complexes (grey lines) were compared pairwise to troponin complex reconstituted with the green fluorescent protein (GFP) conjugated subunits conjugated to the N or the C terminus of GFP as illustrated (red lines). n= 5 error bars are ± SEM. Average Δ values for Ca^{++} sensitivity (pCa_{50}), co-operativity (n_H), minimum and maximum ATPase activity for each paired experiment comparing GFP conjugated troponin to control are detailed in **Supplementary Table 1**.



Supplementary Figure 7. Conjugation of RGECO to human cardiac TnT does not prevent troponin and thin filament reconstitution or myofilament function *in vitro*. RaptorX predicted structures indicated the C terminal T2 head thought to be essential for Tn reconstitution was freely available for interaction with the remaining Tn subunits **(a)**. UV trace data obtained during Tn subunit purification using gel filtration chromatography **(b)**, indicates strong reconstitution and relatively lower abundance of TnI/TnC complex (black arrows) and TnC (red arrows) impurities in Tn reconstitutions containing a 50:50 mixture of WT TnT and RGECO-TnT (purple) compared to 100% WT TnT (grey). Coomassie stained SDS PAGE analysis of the purified Tn complexes **(c)** confirmed robust reconstitution of RGECO TnT (1) compared to WT TnT (2). SDS PAGE analysis of Total (T), supernatant (S) and pellet (P) fractions in actin co-sedimentation assays **(d)** showed that RGECO TnT was able to fully incorporate into reconstituted thin filaments. Myofilament function was assessed using *in vitro* actin activated acto-myosin S1 ATPase assays in **(e)**. The Ca^{++} sensitivity of control (unconjugated) troponin complexes (grey lines) were not significantly different to thin filaments reconstituted with RGECO-TnT (purple lines) in paired experiments **(f)**. n= 5 error bars are ±SEM.

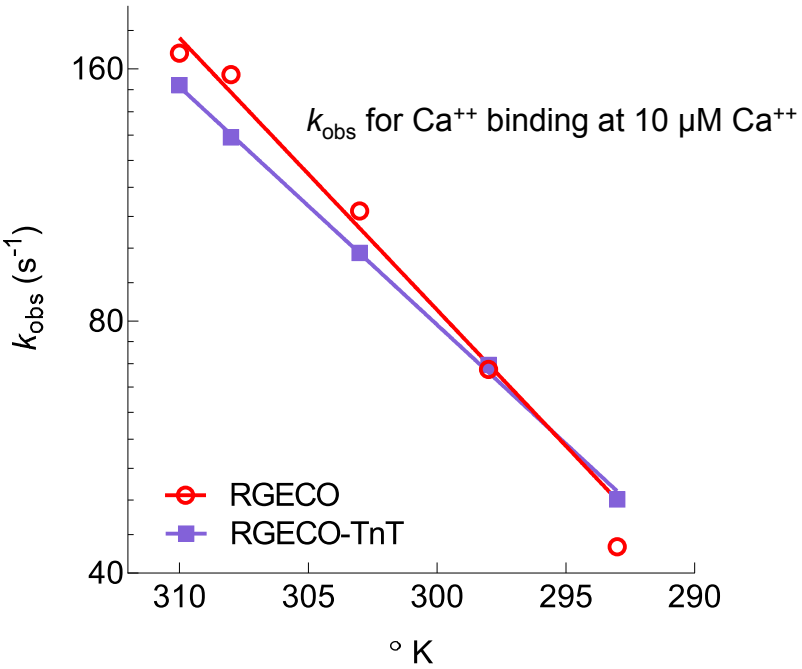


Supplementary Figure 8. The Ca^{++} affinity of RGECO is temperature and Mg^{++} dependent but is not significantly altered by conjugation to TnT. The steady state Ca^{++} binding affinity (K_d) of purified recombinant RGECO protein was determined in the presence of 0, 1.3 and 3.9 mM $MgCl_2$ at 25 and 37 °C **(a)**, the arrowhead denotes the predicted intracellular conditions in the contracting cardiomyocyte. (n=4). There is no significant difference in the fluorescence/pCa relationship **(b)** or the K_d **(c)** between RGECO (Red) and RGECO-TnT (n=4), error bars are ± SEM.



Supplementary Figure 9. Excitation / emission and absorbance spectra of RGECO and RGECO-TnT in the presence and absence of Ca^{++}. Steady state fluorescence excitation, emission (peak excitation (Ex) = 564 nm) / peak emission (Em) = 581nm) and absorbance spectra were obtained at pCa 8.5 (dotted lines) and 4.5 (solid lines) for purified recombinant RGECO **(a and c)** and RGECO-TnT **(b and d)**. All experiments were performed at 37 °C, 1.3 mM $MgCl_2$ pH 7.3. Resultant molar extinction co-efficient, quantum yield, brightness and dynamic range measurements are given in **Supplementary Table 2**.

a



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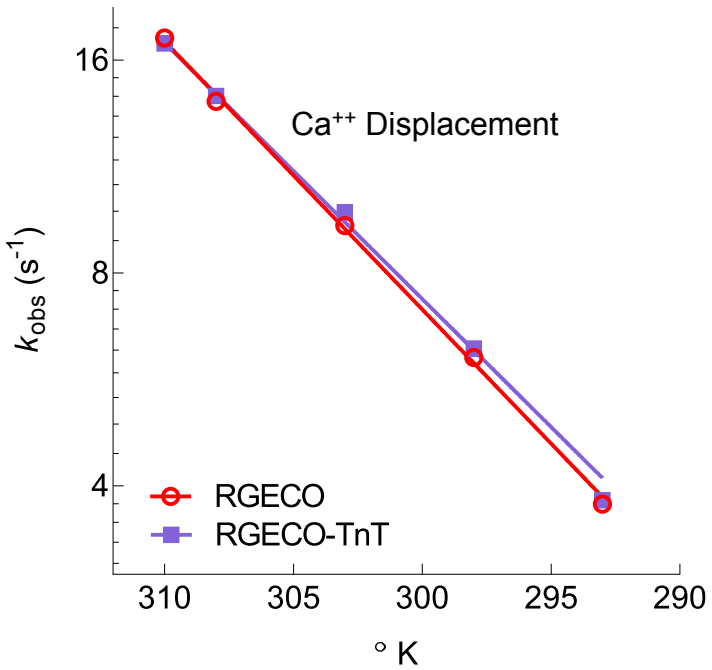
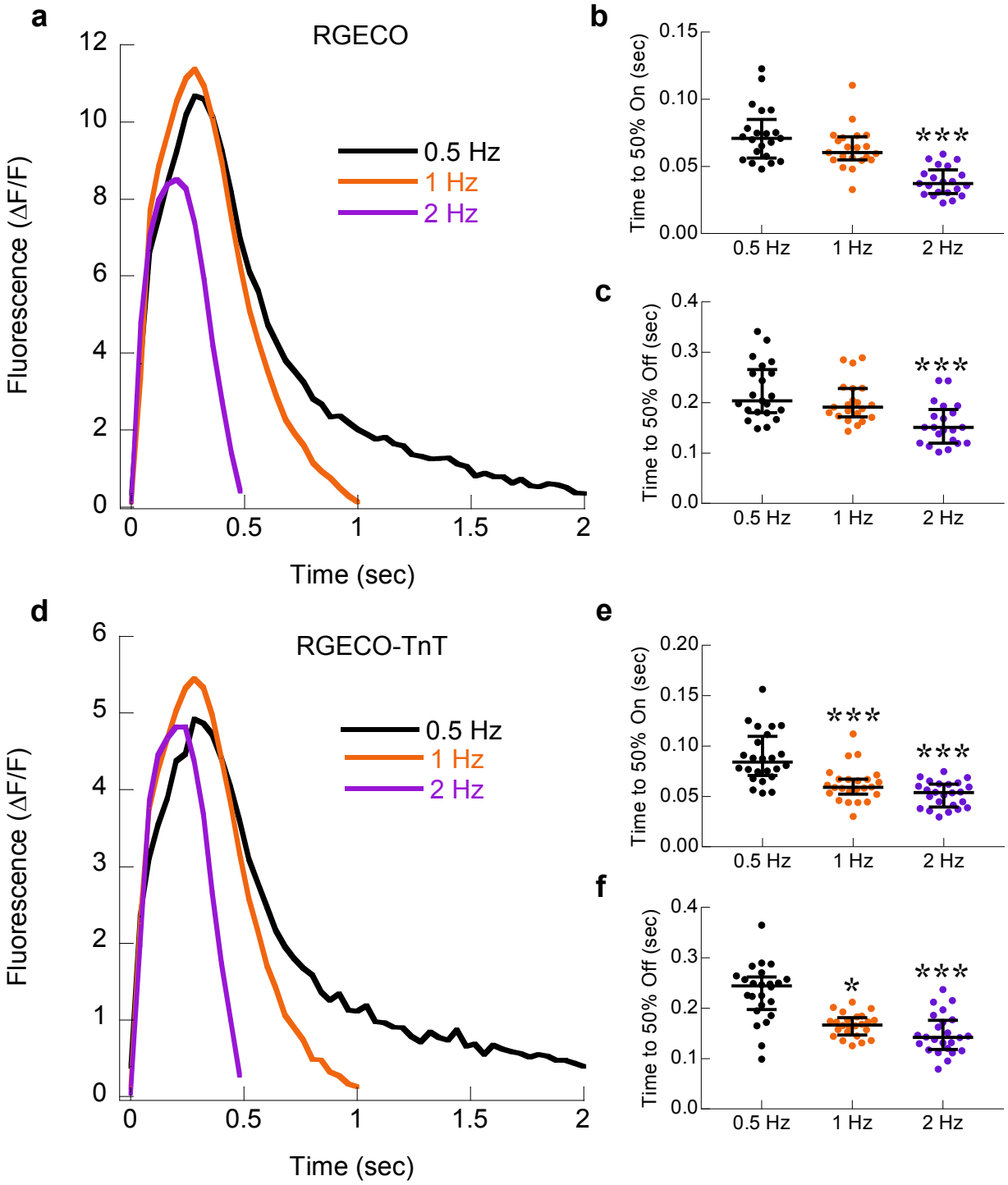


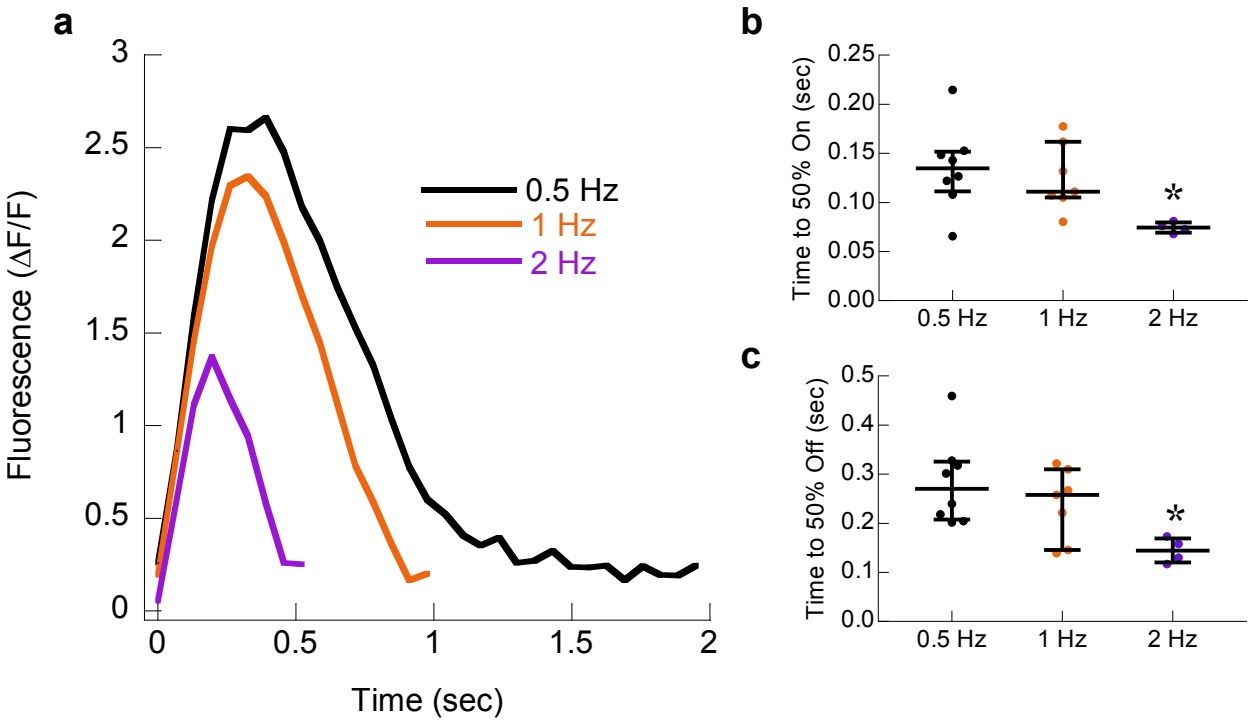
c

	k_{off}/s^{-1}		k_{on}/s^{-1}	
Temp (°C)	**25**	**37**	**25**	**37**
RGECO	6.1	17.2	70.0	153.0
RGECO-TnT	6.3	16.9	70.9	167.0

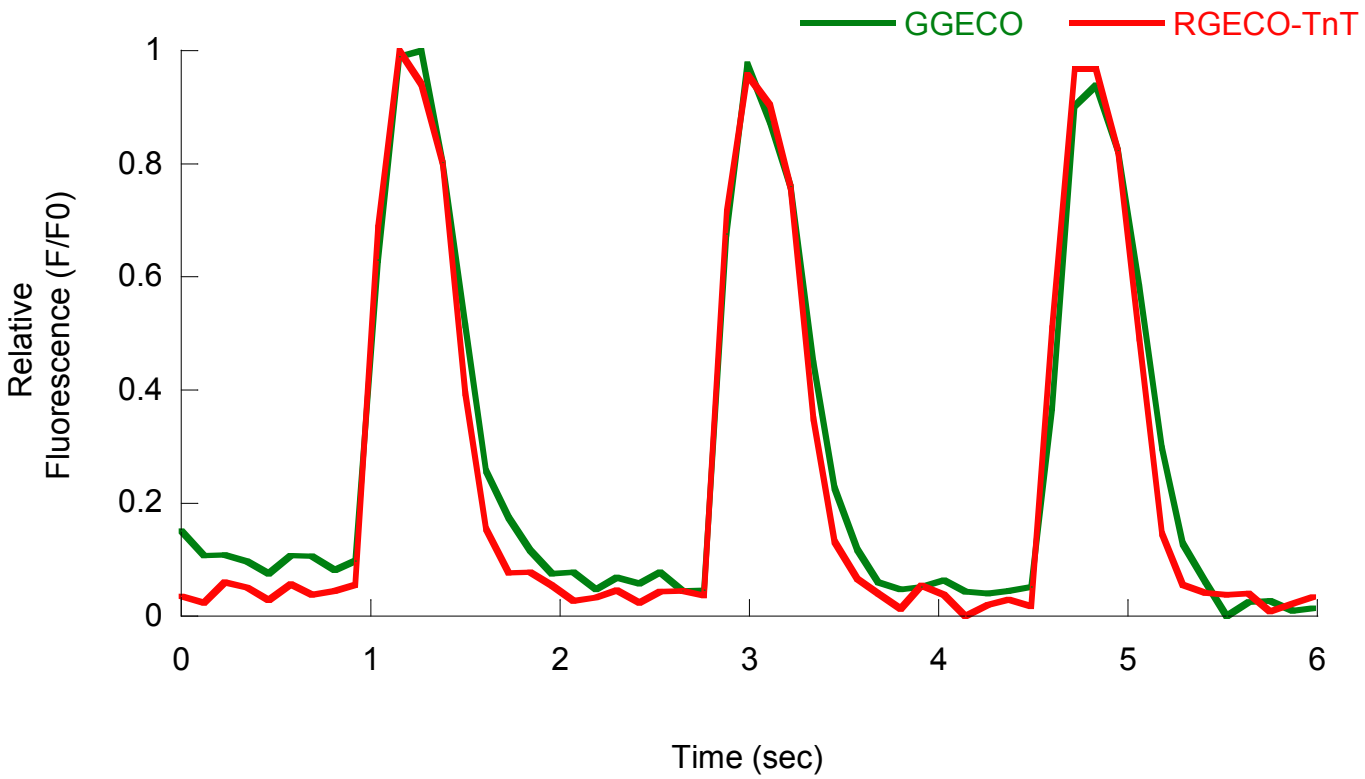
Supplementary Figure 10. Kinetic determination of k_{on} and k_{off} by stopped flow. Arrhenius plots of the observed rate constant of Ca^{++} binding **(a)** and Ca^{++} release rate constant **(b)** as determined by stopped flow measurement of 0.125 μM purified protein. Data for room (25°C) and body (37°C) temperature are shown in **(c).**



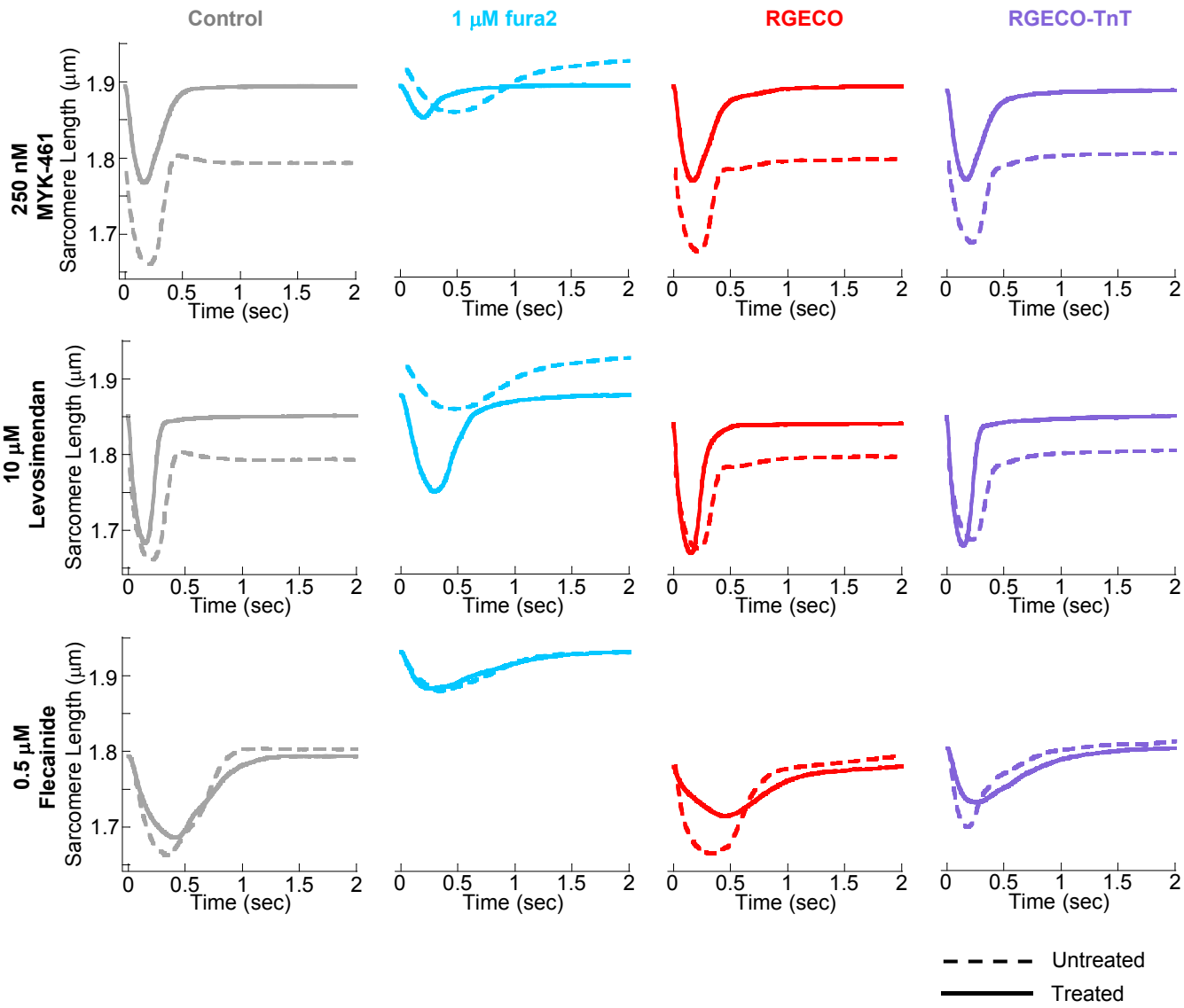
Supplementary Figure 11. RGECO and RGECO-TnT Ca^{++} transients show reverse rate dependence in times to 50% on and 50% off in response to increased pacing frequency in adult GPCMs. Averaged Ca^{++} transients of electrically paced isolated adult cardiomyocytes was used to compare 0.5 Hz (black), 1.0 Hz (orange) and 2 Hz (violet) pacing extracted from fluorescent (F_{581}) video recordings using either RGECO **(a)** or RGECO-TnT **(d).** Adjacent dot plots show distributions of time to 50% binding, and 50% release rates across the pacing range. Shortening of both parameters is apparent for both indicators (RGECO **(b and c)** and RGECO-TnT **(e and f)**). Lines give the median average and error bars are ± interquartile range * = $p<0.05$ and *** = $p<0.001$ using one way ANOVA



Supplementary Figure 12. Reverse rate dependence is seen in RGECO-TnT Ca^{++} transients in response to altered pacing frequency in human iPS-CMs. Averaged Ca^{++} transients of electrically paced cultured iPS-CMs was used to compare 0.5 Hz (black), 1.0 Hz (orange) and 2 Hz (violet) pacing extracted from fluorescent (F_{581}) video recordings using RGECO-TnT **(a).** Adjacent dot plots **(b and c)** show increased pacing frequency reduces the time to 50% Ca^{++} binding and release can be detected. Lines give the median average and error bars are ± interquartile range * = $p<0.05$ using one way ANOVA



Supplementary Figure 13. Myofilament restricted RGECO-TnT Ca^{++} transients (red) obtained simultaneously with cytoplasmic GGECO transients (green) in human iPS-CMs. iPS-CM's were infected with viruses encoding GGECO and the myofilament restricted RGECO-TnT and imaged in a DualView system with two EMCCD cameras to allow simultaneous data acquisition. Relative fluorescence traces obtained from a single cell are shown.



Supplementary Figure 14. Sarcomere length measurements from vGPCM's loaded with three different Ca^{++} indicator strategies (fura2, RGECO or RGECO TnT) in the presence of vehicle or drug (MYK-461, levosimendan or flecainide). Averaged curves from control (dashed line) or drug exposed (solid line) are given for each of the stated indicator, and small molecule combinations. Due to variability inherent in primary cell preparations controls are presented for each condition and the sample split for paired analysis with drug. Absolute numbers for extracted parameters for MYK-461, levosimendan and flecainide treatment are tabulated in **Supplementary Tables 3, 4 and 5** respectively

RGECO-TnT DNA Sequence

ATGGTAGACTCATCACGTCGTAAGTGGAATAAGGCAGGTCACGCAGTCAGAGCTATAGGTCGGCTGAGCTCACCCGTGGT
TTCCGAGCGGATGTACCCCGAGGACGGCGCCCTCAAGAGCGAGATCAAGAAGGGGCTGAGGCTGAAGGACGGCGGCCACT
ACGCCGCCGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACATCGTAGACATCAAGTTG
GACATCGTGTCCCACAACGAGGACTACACCATCGTGGAACAGTGCGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCAT
GGACGAGCTATACAAGGGAGGTACAGGCGGGAGTCTGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGT
TCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCC
TACGAGGCCTTTCAGACCGCTAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCA
GTTCATGTACGGCTCCAAGGCCTACATTAAGCACCCAGCCGACATCCCCGACTACTTCAAGCTGTCCTTCCCCGAGGGCT
TCAGGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCATTATTCACGTTAACCAGGACTCCTCCCTGCAGGACGGCGTA
TTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCCCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGA
GGCTACGCGTGACCAACTGACTGAGGAGCAGATCGCAGAATTTAAAGAGGCTTTCTCCCTATTTGACAAGGACGGGGATG
GGACGATAACAACCAAGGAGCTGGGGACGGTGATGCGGTCTCTGGGGCAGAACCCCACAGAAGCAGAGCTGCAGGACATG
ATCAATGAAGTAGATGCCGACGGTGACGGCACATTCGACTTCCCTGAGTTCCTGACGATGATGGCAAGAAAAATGAATGA
CACAGACAGTGAAGAGGAAATTAGAGAAGCGTTCCGCGTGTTTGATAAGGACGGCAATGGCTACATCGGCGCAGCAGAGC
TTCGCCACGTGATGACAGACCTTGGAGAGAAGTTAACAGATGAGGAGGTTGATGAAATGATCAGGGTAGCAGACATCGAT
GGGGATGGTCAGGTAAACTACGAAGAGTTTGTCCAAATGATGACAGCGAAGGGATCCATGTCTGACATAGAAGAGGTGGT
GGAAGAGTACGAGGAGGAGGAGCAGGAAGAAGCAGCTGTTGAAGAGCAGGAGGAGGCAGCGGAAGAGGATGCTGAAGCAG
AGGCTGAGACCGAGGAGACCAGGGCAGAAGAAGATGAAGAAGAAGAGGAAGCAAAGGAGGCTGAAGATGGCCCAATGGAG
GAGTCCAAACCAAAGCCCAGGTCGTTCATGCCCAACTTGGTGCCTCCCAAGATCCCCGATGGAGAGAGAGTGGACTTTGA
TGACATCCACCGGAAGCGCATGGAGAAGGACCTGAATGAGTTGCAGGCGCTGATTGAGGCTCACTTTGAGAACAGGAAGA
AAGAGGAGGAGGAGCTCGTTTCTCTCAAAGACAGGATCGAGAGACGTCGGGCAGAGCGGGCCGAGCAGCAGCGCATCCGG
AATGAGCGGGAGAAGGAGCGGCAGAACCGCCTGGCTGAAGAGAGGGCTCGACGAGAGGAGGAGGAGAACAGGAGGAAGGC
TGAGGATGAGGCCCGGAAGAAGAAGGCTTTGTCCAACATGATGCATTTTGGGGGTTACATCCAGAAGCAGGCCCAGACAG
AGCGGAAAAGTGGGAAGAGGCAGACTGAGCGGGAAAAGAAGAAGAAGATTCTGGCTGAGAGGAGGAAGGTGCTGGCCATT
GACCACCTGAATGAAGATCAGCTGAGGGAGAAGGCCAAGGAGCTGTGGCAGAGCATCTATAACTTGGAGGCAGAGAAGTT
CGACCTGCAGGAGAAGTTCAAGCAGCAGAAATATGAGATCAATGTTCTCCGAAACAGGATCAACGATAACCAGAAAGTCT
CCAAGACCCGCGGGAAGGCTAAAGTCACCGGGCGCTGGAAATAG

RGECO-TnT amino acid Sequence

MVDSSRRKWNKAGHAVRAIGRLSSPVVSERMYPEDGALKSEIKKGLRLKDGGHYAAEVKTTYKAKKPVQLPGAYIVDIKL
DIVSHNEDYTIVEQCERAEGRHSTGGMDELYKGGTGGSLVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRP
YEAFQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYIKHPADIPDYFKLSFPEGFRWERVMNFEDGGIIHVNQDSSLQDGV
FIYKVKLRGTNFPPDGPVMQKKTMGWEATRDQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDM
INEVDADGDGTFDFPEFLTMMARKMNDTDSEEEIREAFRVFDKDGNGYIGAAELRHVMTDLGEKLTDEEVDEMIRVADID
GDGQVNYEEFVQMMTAKGSMSDIEEVVEEYEEEEQEEAAVEEQEEAAEEDAEAEAETEETRAEEDEEEEEAKEAEDGPME
ESKPKPRSFMPNLVPPKIPDGERVDFDDIHRKRMEKDLNELQALIEAHFENRKKEEEELVSLKDRIERRRAERAEQQRIR
NEREKERQNRLAEERARREEEENRRKAEDEARKKKALSNMMHFGGYIQKQAQTERKSGKRQTEREKKKKILAERRKVLAI
DHLNEDQLREKAKELWQSIYNLEAEKFDLQEKFKQQKYEINVLRNRINDNQKVSKTRGKAKVTGRWK.

Supplementary Figure 15. DNA and amino acid sequence of RGECO-TnT. Sequence highlighted red is RGECO, purple is human cardiac TnT and black is a BamH1 cloning site which corresponds to an additional GS linker sequence in the translated amino acid.

	GFP-TnT	TnT-GFP	GFP-TnC	TnC-GFP
ΔpCa_{50}	-0.031±0.018	-0.149±0.056	0.564±0.269	-0.369±0.125
Δn_H	0.980±0.691	7.308±3.703	0.8649±0.598	-0.867±0.912
ΔMin (sec^{-1})	0.548±0.219	0.185±0.089	**6.551±0.969 *****	**5.382±1.874 ***
ΔMax (sec^{-1})	0.686±0.588	**-2.958±0.399 ***	1.479±0.798	**1.439±0.545 ***

Supplementary Table 1. Extracted parameters from *in vitro* actin activated acto-myosin S1 ATPase assays performed to investigate myofilament function in the presence of GFP conjugates of the troponin complex. Δ values from paired experimental comparisons for pCa_{50}, n_H, maximum and minimum activity (sec^{-1}) with standard error and significance taken from ATPase assays in **Supplementary Figure 6**. n=5, significance values comparing unconjugated with GFP conjugated troponin are $p<0.001$=***, $p<0.01$=** and $p<0.05$=*.

	RGECO	RGECO-TnT
K_d at 25 °C (nM)	1607	1186
K_d at 37 °C (nM)	860	764
Quantum Yield pCa 4.5 (Φ)	0.20	0.33
Quantum Yield pCa 8.5 (Φ)	0.06	0.11
Molar Extinction coefficent pCa 4.5 (ε (mM^{-1}•cm^{-1})) (565 nm)	32.39	34.53
Molar Extinction coefficent pCa 8.5 (ε (mM^{-1}•cm^{-1})) (565 nM)	5.17	6.64
Molar Extinction coefficent pCa 4.5 (ε (mM^{-1}•cm^{-1})) (455 nm)	4.16	6.18
Molar Extinction coefficent pCa 8.5 (ε (mM^{-1}•cm^{-1})) (455 nM)	11.25	14.11
Brightness pCa 4.5 (mM^{-1}•cm^{-1})	6.48	11.30
Brightness pCa 8.5 (mM^{-1}•cm^{-1})	0.31	0.73
Intensity change ± Ca^{2+}	10.18X	10.33X

Supplementary Table 2. Extracted parameters from Fluorescence and absorbance spectra and kinetic experiments at 1.3mM $MgCl_2$. Brightness is defined as the product of ε and Φ.

a

Sensor	Drug	*n*	fura2 Fluorescence Amplitude ($F_{365/380}$) / GECO Peak Intensity ($\Delta F_{581}/F$)	Time to 50% Ca^{++} Binding (sec)	Time to 50% Ca^{++} Release (sec)	Basal Fluorescence ($F_{365/380}$)
1 μM fura2	**DMSO**	*22*	0.282±0.019	0.035±0.005	0.278±0.014	1.091±0.026
	250 nM MYK-461	*27*	0.270±0.016	0.033±0.003	**0.213±0.020 ***	**1.297±0.020 *****
RGECO	**DMSO**	*79*	12.50±0.697	0.079±0.003	0.382±0.007	-
	250 nM MYK-461	*84*	**10.65±0.438 ***	0.073±0.003	**0.222±0.005 *****	-
RGECO-TnT	**DMSO**	*84*	7.140±0.280	0.079±0.003	0.268±0.008	-
	250 nM MYK-461	*68*	**5.728±0.265 *****	**0.048±0.002 *****	**0.228±0.009 *****	-

b

Sensor	Drug	*n*	Fractional Sarcomere Shortening (%)	Diastolic Sarcomere Length (μm)	Time to 50% Contraction (sec)	Time to 50% Relaxation (sec)
Control	**DMSO**	*26*	7.456±0.381	1.791±0.015	0.056±0.002	0.120±0.005
	250 nM MYK-461	*36*	7.177±0.500	**1.892±0.007 *****	0.063±0.004	**0.102±0.006 ***
1 μM fura2	**DMSO**	*20*	4.351±0.534	1.925±0.014	0.231±0.018	0.395±0.031
	250 nM MYK-461	*18*	**2.409±0.464 *****	**1.894±0.009 *****	**0.106±0.014 *****	**0.153±0.014 *****
RGECO	**DMSO**	*33*	7.094±0.351	1.795±0.012	0.067±0.005	0.147±0.011
	250 nM MYK-461	*41*	7.188±0.450	**1.891±0.006 *****	0.072±0.005	0.126±0.010
RGECO-TnT	**DMSO**	*37*	6.798±0.348	1.804±0.014	0.073±0.005	0.164±0.013
	250 nM MYK-461	*44*	6.788±0.468	**1.886±0.006 *****	0.071±0.004	**0.130±0.011 ***

Supplementary table 3. Extracted parameters from Ca^{++} transients and unloaded sarcomere shortening curves upon the application of 250 nM MYK-461. Extracted values from paired experimental comparisons of MYK-461 to control in table **(a),** were taken from Ca^{++} transients in **Figure 2a**. **(b)** gives parameters from unloaded sarcomere shortening in **Supplementary Figure 14.** n = total cell number , standard error and significance were taken from RGECO and RGECO-TnT Ca^{++} transients in Figure 2B column 2 and 3 respectively. Significance values (highlighted red or blue (if directionally opposite to control experiments)) comparing pre and post treatment are $p<0.001$=***, $p<0.01$=** and $p<0.05$=*.

a

Sensor	Drug	*n*	fura2 Fluorescence Amplitude ($F_{365/380}$) / GECO Peak Intensity ($\Delta F_{581}/F$)	Time to 50% Ca^{++} Binding (sec)	Time to 50% Ca^{++} Release (sec)	Basal Fluorescence ($F_{365/380}$)
1 μM fura2	DMSO	*22*	0.286±0.018	0.035±0.005	0.287±0.015	1.105±0.026
	10 μM Levosimendan	*32*	**0.397±0.019 *****	0.038±0.003	**0.230±0.008 ****	1.146±0.022
RGECO	DMSO	*77*	10.24±0.534	0.078±0.003	0.329±0.008	-
	10 μM Levosimendan	*83*	**15.12±0.629 *****	0.071±0.003	**0.245±0.004 *****	-
RGECO-TnT	DMSO	*74*	6.340±0.285	0.069±0.004	0.230±0.007	-
	10 μM Levosimendan	*76*	**18.94±1.476 *****	**0.053±0.002 *****	**0.208±0.009 ***	-

b

Sensor	Drug	*n*	Fractional Sarcomere Shortening (%)	Diastolic Sarcomere Length (μm)	Time to 50% Contraction (sec)	Time to 50% Relaxation (sec)
Control	DMSO	*24*	7.563±0.561	1.791±0.015	0.056±0.002	0.122±0.005
	10 μM Levosimendan	*36*	**9.266±0.409 ****	**1.849±0.010 ****	**0.045±0.002 *****	**0.071±0.003 *****
1 μM fura2	DMSO	*22*	4.392±0.491	1.926±0.015	0.232±0.017	0.386±0.028
	10 μM Levosimendan	*27*	**7.286±0.613 ****	**1.875±0.011 *****	**0.139±0.008 *****	**0.193±0.014 *****
RGECO	DMSO	*30*	7.001±0.352	1.794±0.012	0.068±0.005	0.151±0.011
	10 μM Levosimendan	*43*	**9.266±0.409 *****	**1.838±0.006 ****	**0.046±0.002 ****	**0.071±0.003 *****
RGECO-TnT	DMSO	*34*	6.839±0.336	1.795±0.014	0.073±0.005	0.161±0.013
	10 μM Levosimendan	*42*	**9.443±0.40 *****	**1.848±0.009 ****	**0.045±0.002 *****	**0.073±0.003 *****

Supplementary table 4. Extracted parameters from Ca^{++} transients and unloaded sarcomere shortening curves upon the application of 10 μM Levosimendan. Extracted values from paired experimental comparisons of Levosimendan to control in table **(a),** were taken from Ca^{++} transients in **Figure 2a**. **(b)** gives parameters from unloaded sarcomere shortening in **Supplementary Figure 14.** n = total cell number , standard error and significance were taken from RGECO and RGECO-TnT Ca^{++} transients in Figure 2b column 2 and 3 respectively. Significance values (highlighted red or blue (if directionally to opposite control experiments)) comparing pre and post treatment are $p<0.001$=***, $p<0.01$=** and $p<0.05$=*.

a

Sensor	Drug	*n*	fura2 Fluorescence Amplitude ($F_{365/380}$) / GECO Peak Intensity ($\Delta F_{581}/F$)	Time to 50% Ca^{++} Binding (sec)	Time to 50% Ca^{++} Release (sec)	Basal Fluorescence ($F_{365/380}$)
1 μM fura2	DMSO	*24*	0.257±0.027	0.037±0.004	0.302±0.023	1.086±0.018
	0.5 μM Flecainide	*24*	**0.161±0.013 *****	0.039±0.006	**0.377±0.027 ***	1.161±0.012
RGECO	DMSO	*50*	13.64±0.564	0.074±0.004	0.276±0.007	-
	0.5 μM Flecainide	*52*	15.13±0.739	0.074±0.003	**0.316±0.007 *****	-
RGECO-TnT	DMSO	*78*	8.558±0.323	0.070±0.003	0.243±0.007	-
	0.5 μM Flecainide	*68*	**6.815±0.291 *****	0.065±0.003	**0.267±0.007 ***	-

b

Sensor	Drug	*n*	Fractional Sarcomere Shortening (%)	Diastolic Sarcomere Length (μm)	Time to 50% Contraction (sec)	Time to 50% Relaxation (sec)
Control	DMSO	*26*	8.094±0.398	1.799±0.010	0.125±0.007	0.211±0.011
	0.5 μM Flecainide	*24*	**6.404±0.354 ****	1.789±0.012	**0.160±0.014 ***	**0.262±0.015 ****
1 μM fura2	DMSO	*25*	3.274±0.388	1.932±0.013	0.150±0.015	0.348±0.019
	0.5 μM Flecainide	*22*	3.539±0.343	1.926±0.015	0.157±0.021	**0.293±0.022 *****
RGECO	DMSO	*25*	7.715±0.556	1.791±0.009	0.084±0.006	0.217±0.010
	0.5 μM Flecainide	*26*	**3.798±0.272 *****	1.778±0.010	**0.166±0.027 ****	**0.348±0.016 *****
RGECO-TnT	DMSO	*28*	6.703±0.590	1.800±0.012	0.069±0.004	0.224±0.022
	0.5 μM Flecainide	*24*	**3.834±0.470 *****	1.792±0.014	**0.081±0.006 ***	**0.369±0.022 *****

Supplementary table 5. Extracted parameters from Ca^{++} transients and unloaded sarcomere shortening curves upon the application of 0.5 μM Flecainide. Extracted values from paired experimental comparisons of Flecainide to control in table **(a),** were taken from Ca^{++} transients in **Figure 2a**. **(b)** gives parameters from unloaded sarcomere shortening in **Supplementary Figure 14.** n = total cell number , standard error and significance were taken from RGECO and RGECO-TnT Ca^{++} transients in Figure 2B column 2 and 3 respectively. Significance values (highlighted red or blue (if directionally opposite to control experiments)) comparing pre and post treatment are $p<0.001$=***, $p<0.01$=** and $p<0.05$=*.