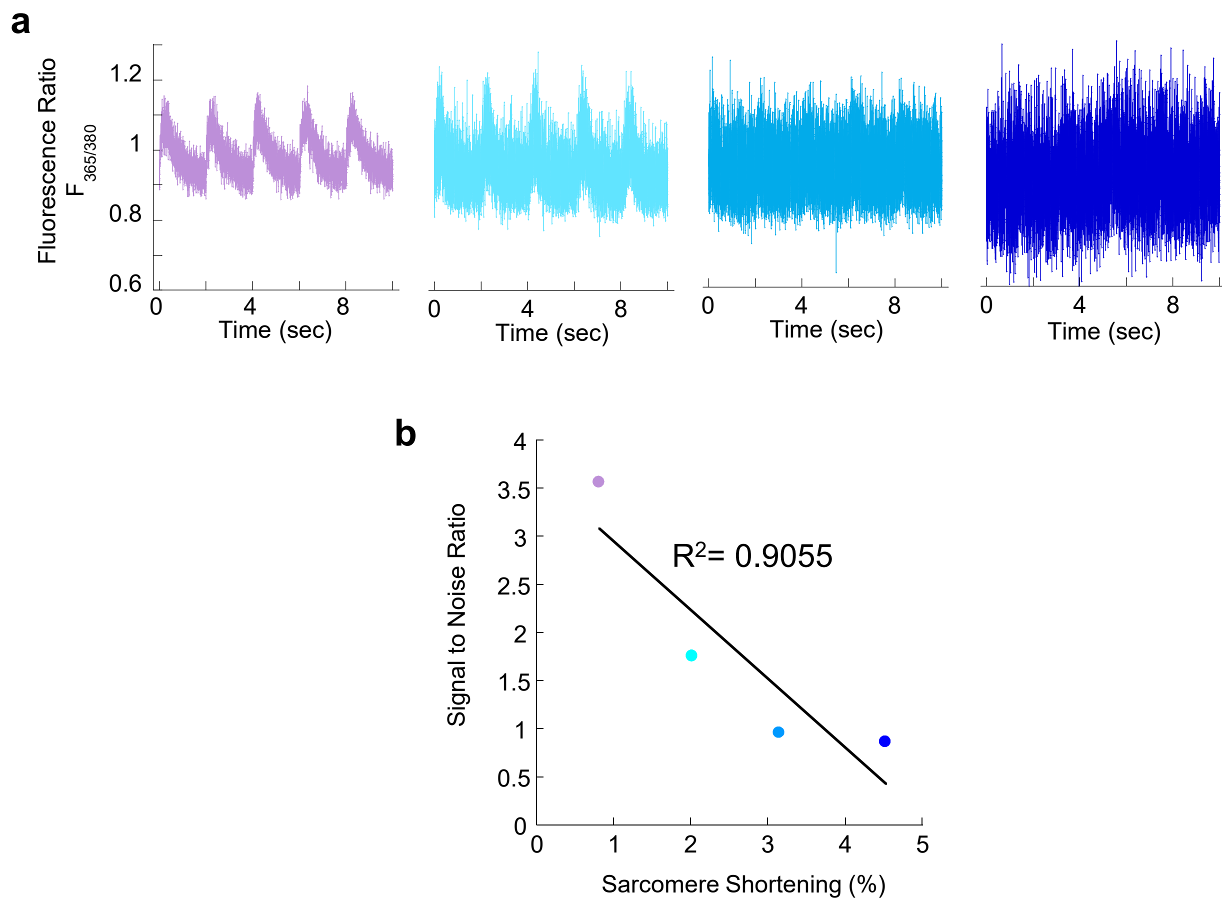
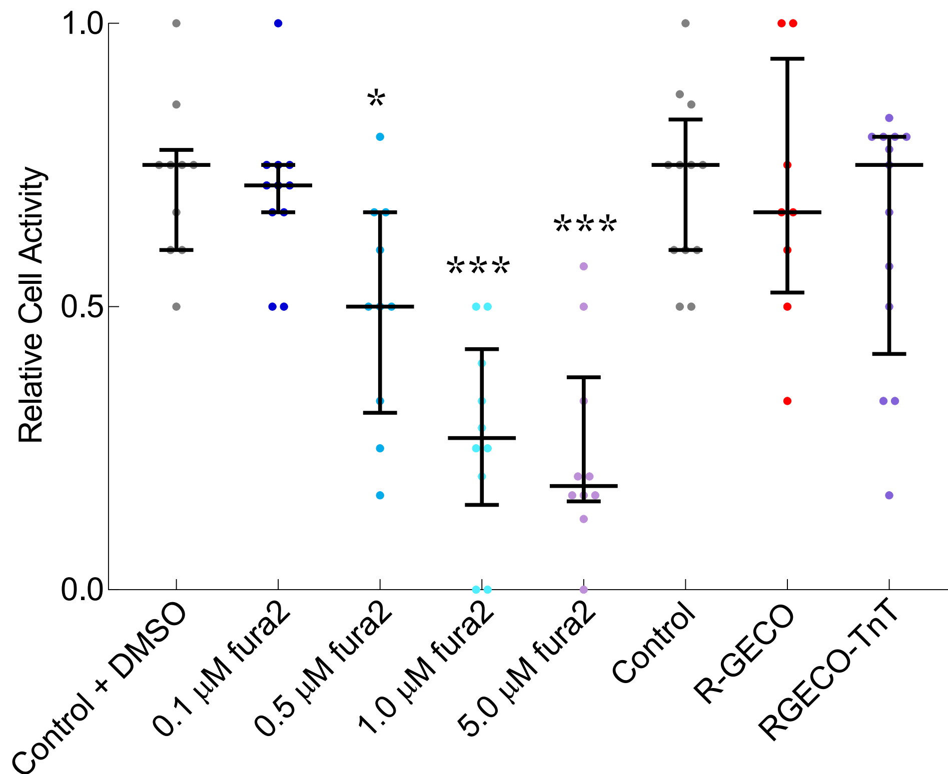
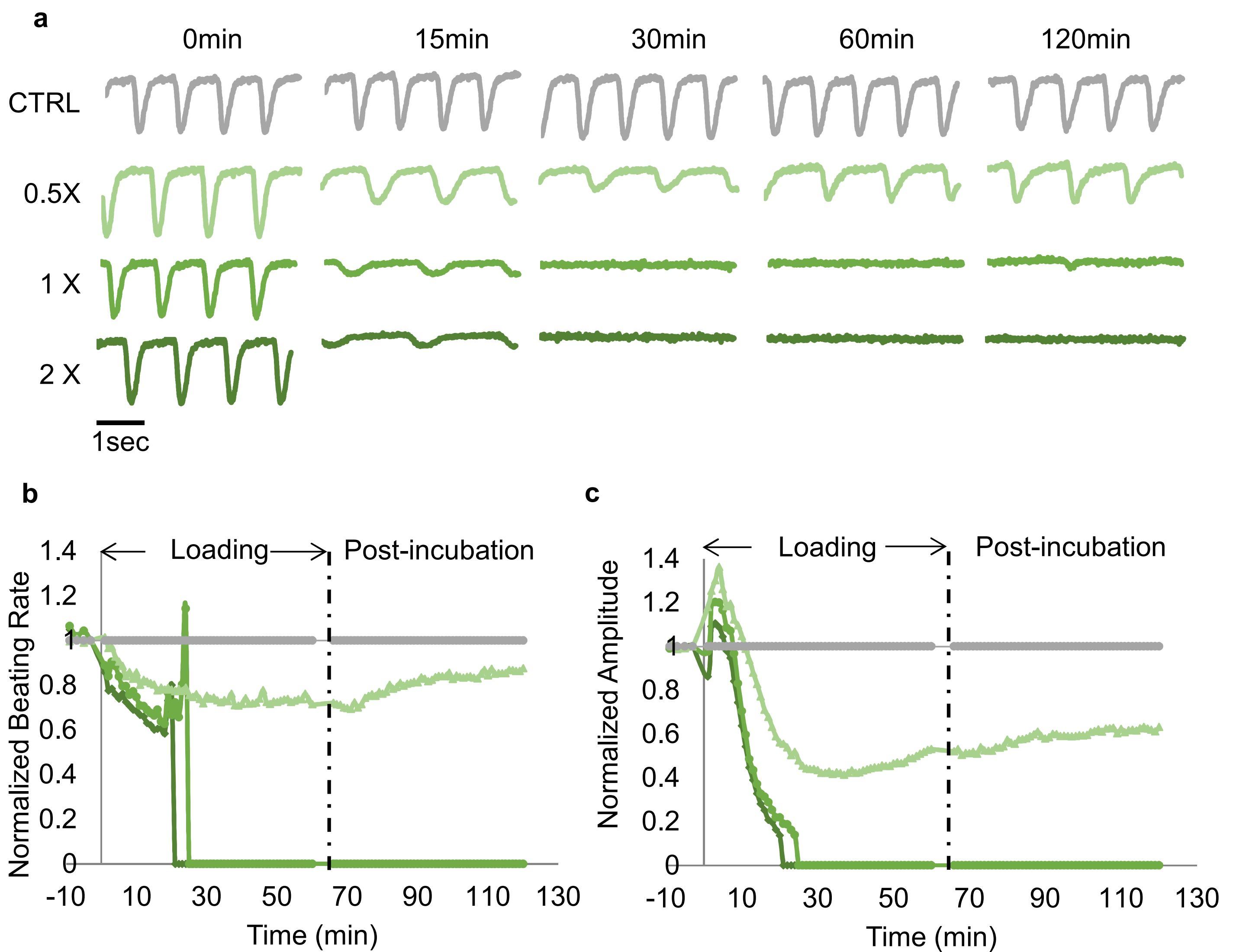
Supplementary Information for "Measurement of myofilament calcium in living cardiomyocytes using a targeted genetically encoded indicator"



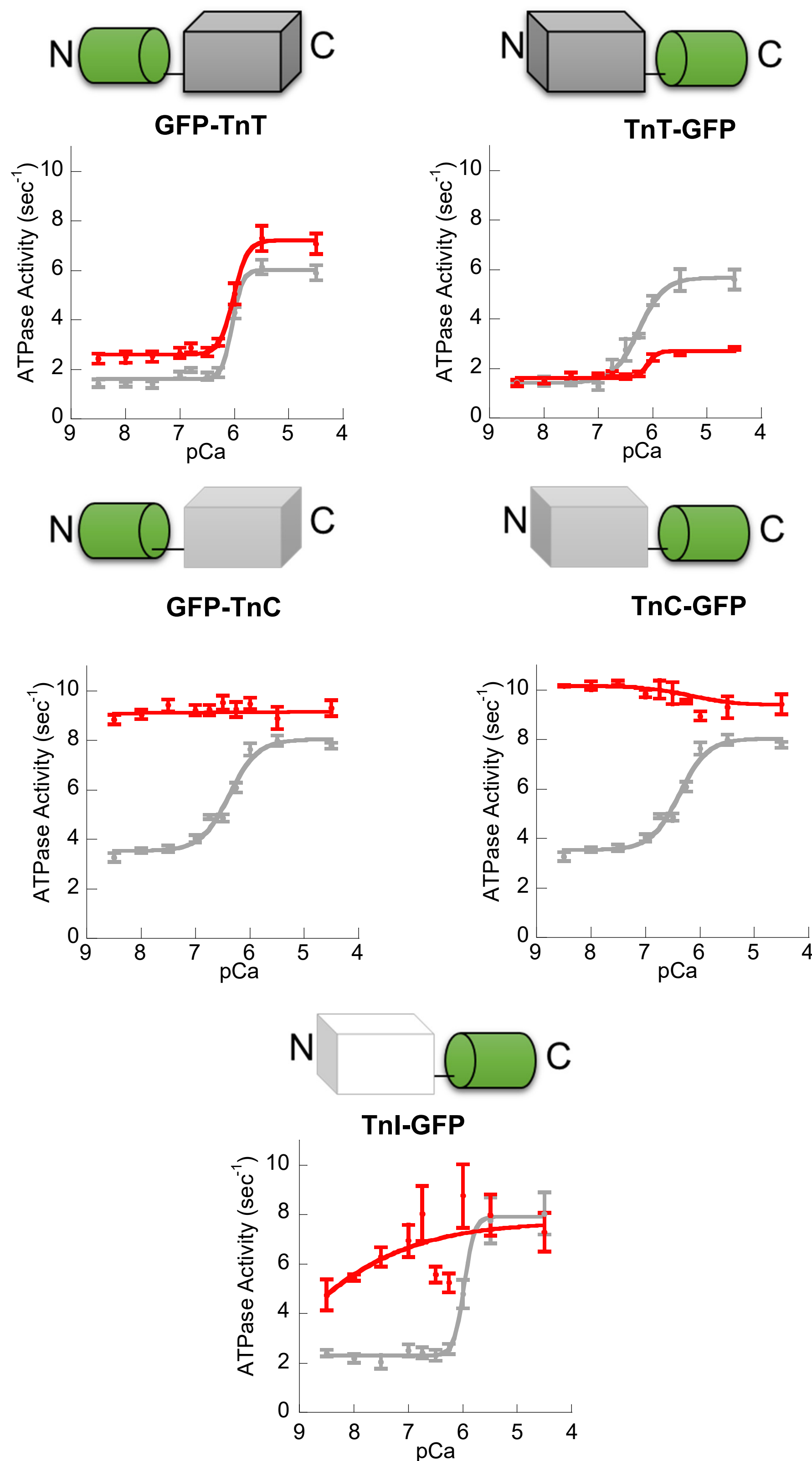
Supplementary Figure 1. fura2-AM ester reduces sarcomere shortening vs fluorescence signal to noise ratio in a dose dependent manner. Example traces taken from the acquisition of Ca^{++} dependent dynamic signal ($F_{365/380}$) in **(a)**, Comparisons of control (grey) to cells loaded with 0.1µM (royal blue), 0.5µM (blue), 1.0µM (teal) or 5.0µM (mauve) fura2-AM ester showed that it was necessary to load cardiomyocytes with >1µM fura2 in order to observe a transient signal. Pairwise sarcomere shortening magnitudes were taken from **figure 1b** and plotted vs ($F_{365/380}$) signal to noise ratios derived from the dynamic signal. A clear negatively correlated relationship exists between signal-to-noise of the Ca^{++} transient and fractional shortening of the sarcomere in vGPCM's **(b)**. (n=23-26).



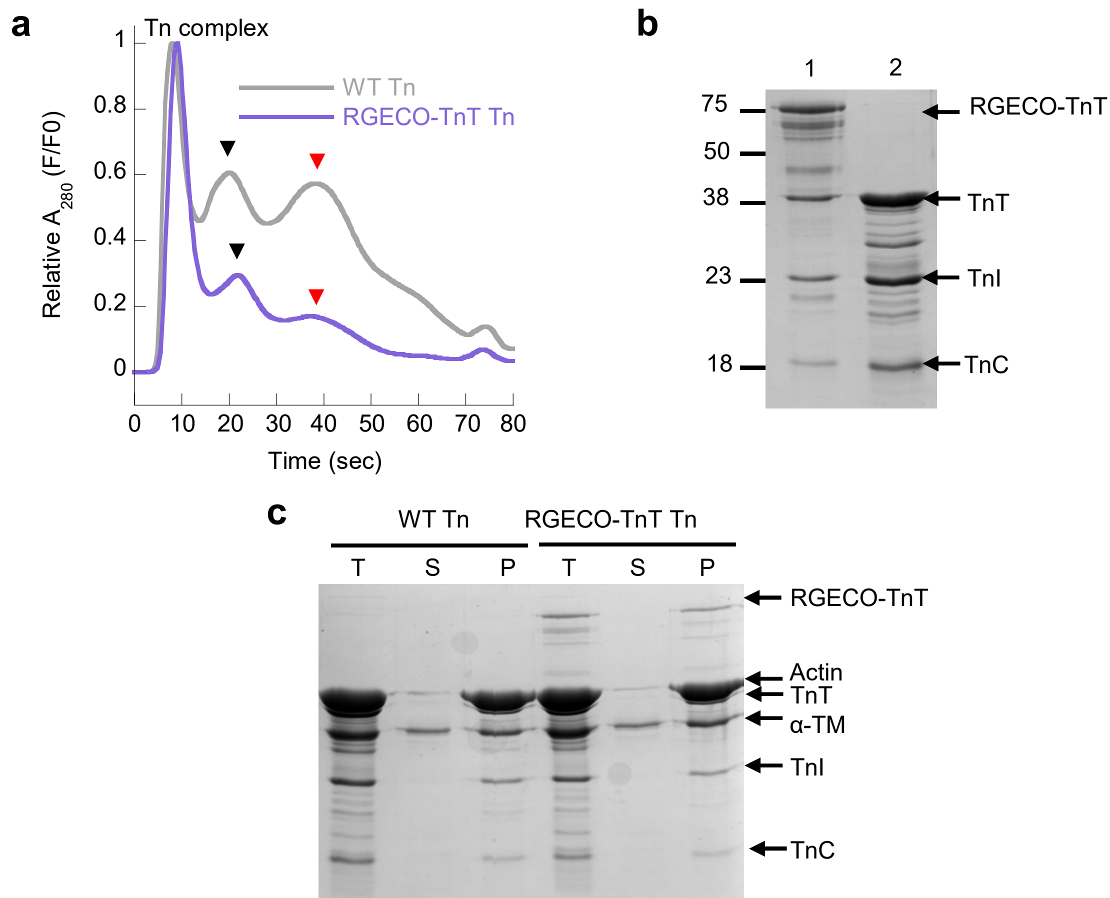
Supplementary Figure 2. fura2-AM ester reduces the relative contractile activity of human iPS-CMs whilst RGECO or RGECO-TnT do not. White light observation of singularised iPS-CMs was used to determine the fraction obviously contracting within a field of view over a two minute interval. Control (with and with out DMSO) (grey) were compered to cells loaded with 0.1µM (royal blue), 0.5µM (blue), 1.0µM (teal) or 5.0µM (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 0.5µM. Lines give the median average and error bars are ± interquartile range * = $p<0.05$ and *** = $p<0.001$ using one way ANOVA.



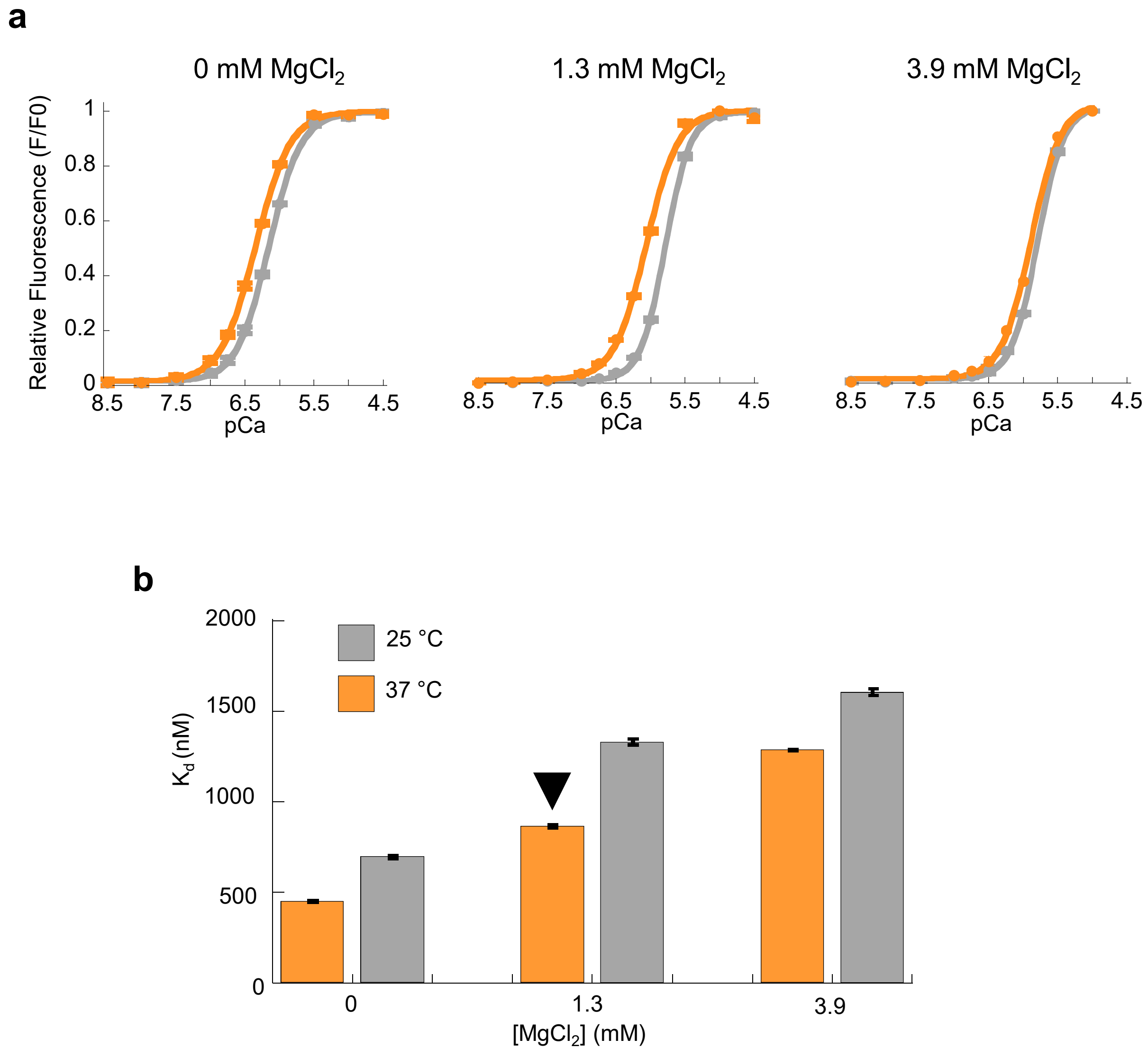
Supplementary Figure 3. The Ca^{++} dye FLIPR Calcium 5 reduces the contractile activity of human iPS-CMs. Label-free impedance monitoring to quantitatively evaluate iPSC-CM contractility in real-time, in the absence of light was undertaken with the Xcelligence RTCA Cardio system using 20,000 CDI cells 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.



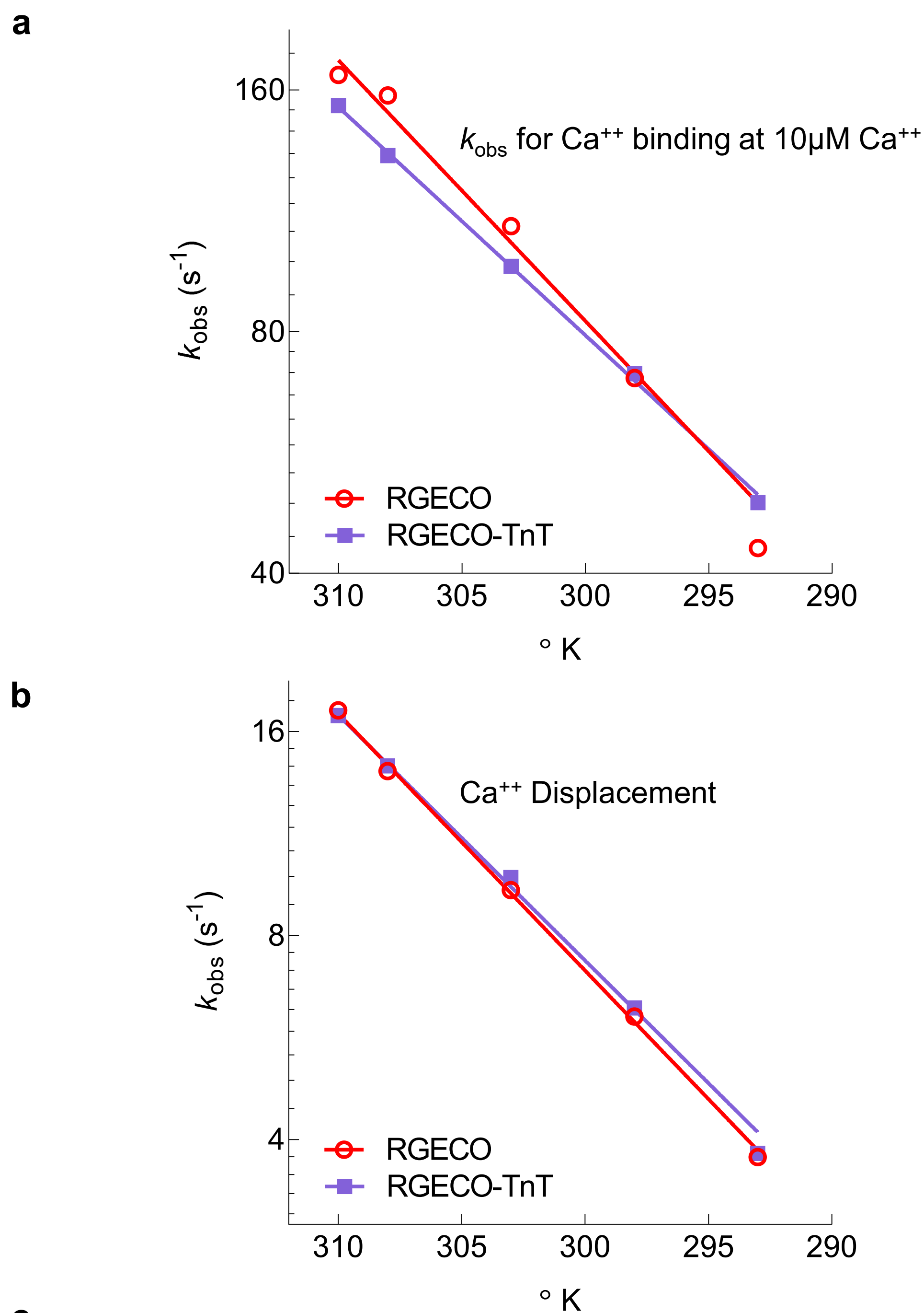
Supplementary Figure 4. The effect of GFP conjugation to the N and C terminus of troponin complex proteins on myofilament function. Myofilament function was assessed using *in vitro* actin activated acto-myosin S1 ATPase assays. Control (unconjugated) troponin complexes (grey lines) were compared pairwise 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**. Only the fusion to the N-terminal of Troponin-T (top left) appears well tolerated biochemically.



Supplementary Figure 5. Conjugation of RGECO to human cardiac TnT does not prevent troponin and thin filament reconstitution. UV trace data obtained during Tn subunit purification using gel filtration chromatography **(a)**, 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 **(b)** 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 **(c)** showed that RGECO TnT was able to fully incorporate into reconstituted thin filaments.

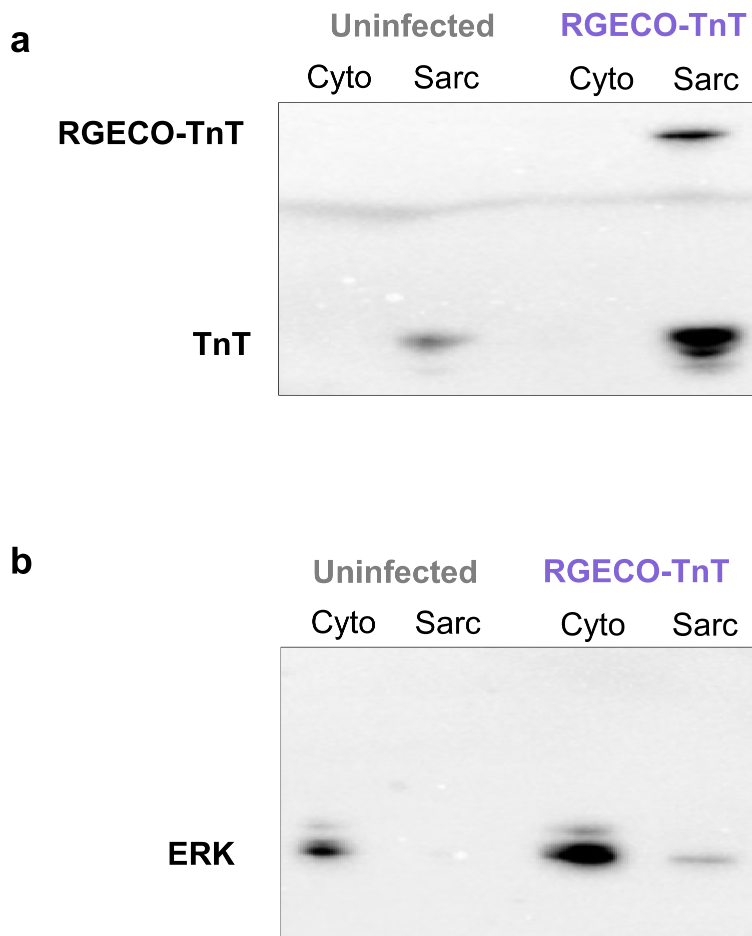


Supplementary Figure 6. The Ca^{++} affinity of RGECO is temperature and Mg^{++} dependent. The fluorescence : pCa relationship **(a)** was used to calculated the steady state Ca^{++} binding affinity (K_d) of purified recombinant RGECO protein in the presence of 0, 1.3 and 3.9mM $MgCl_2$ at 25 and 37°C **(b)**, the arrowhead denotes the predicted intracellular conditions in the contracting cardiomyocyte. (n=4), error bars are ± SEM.

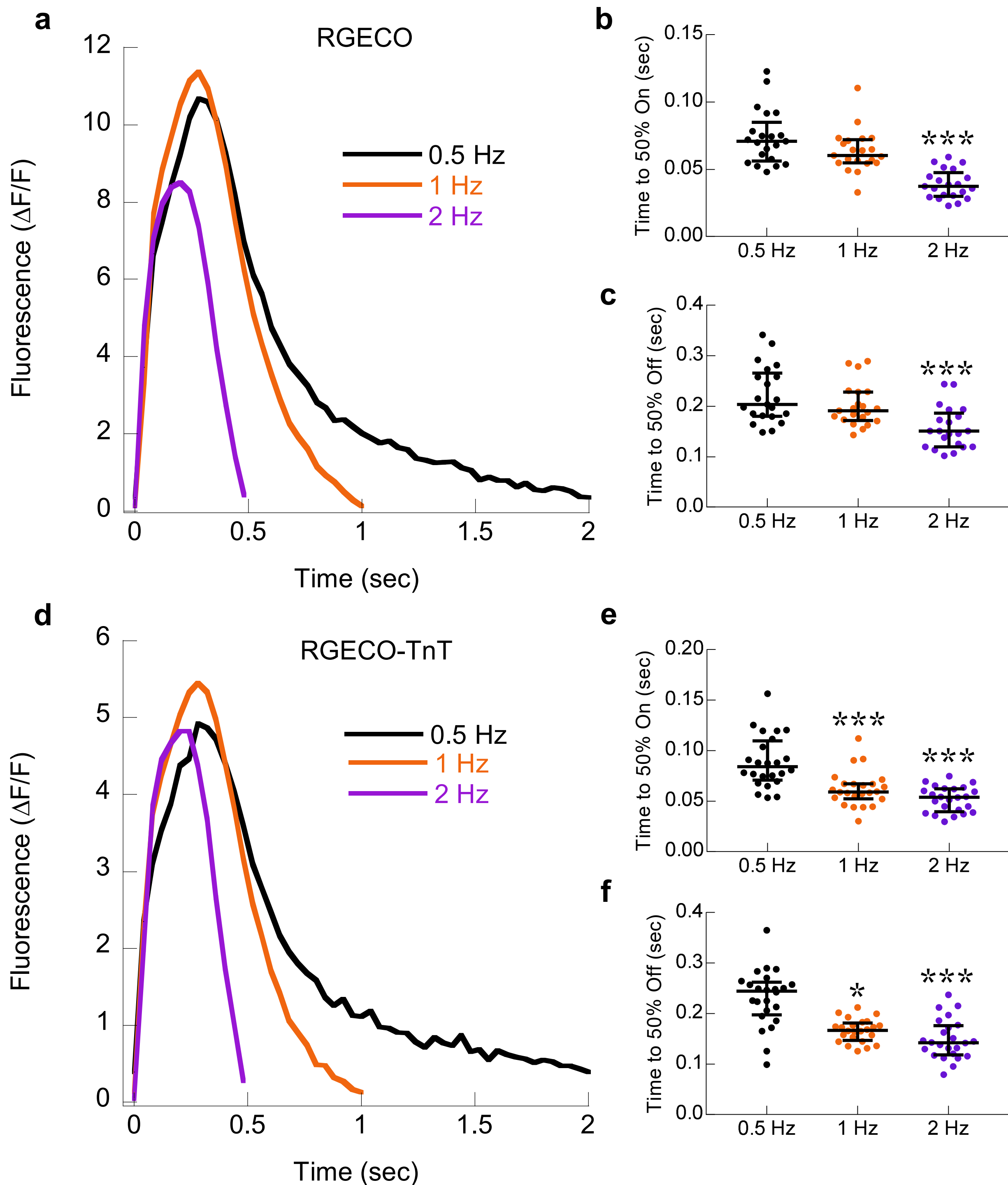


	k_{off}/s^{-1}		k_{on}/s^{-1}	
Temp (°C)	**25**	**37**	**25**	**37**
RGECO	6.1	17.2	70.0	153.0
RGECO-TnT	6.3	16.9	70.9	167.0

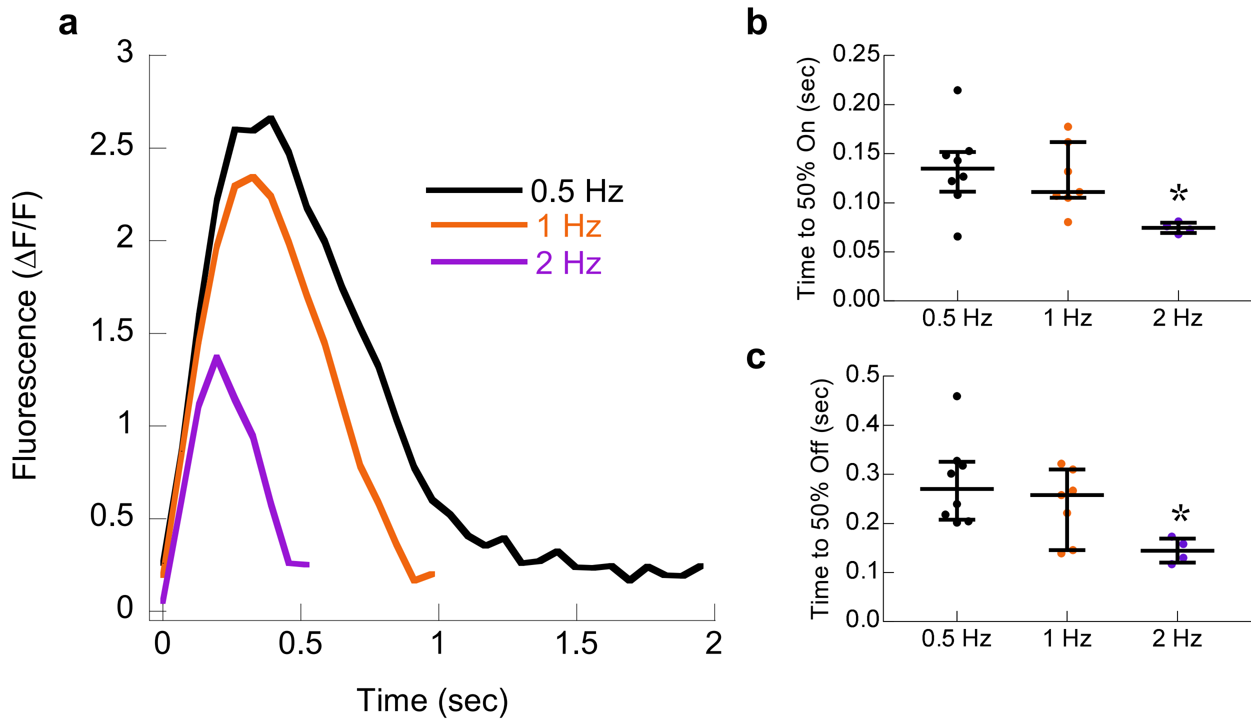
Supplementary Figure 7. Kinetic determination of k_{on} and k_{off} for RGECO-TnT compared to RGECO by stopped flow. Arrhenius plots of the observed rate constant of Ca^{++} binding **(a)** and Ca^{++} release rate constant **(b)** as determined by stopped flow measurement of 0.125μM purified protein. Data for room (25°C) and body (37°C) temperature are shown in **(c).**



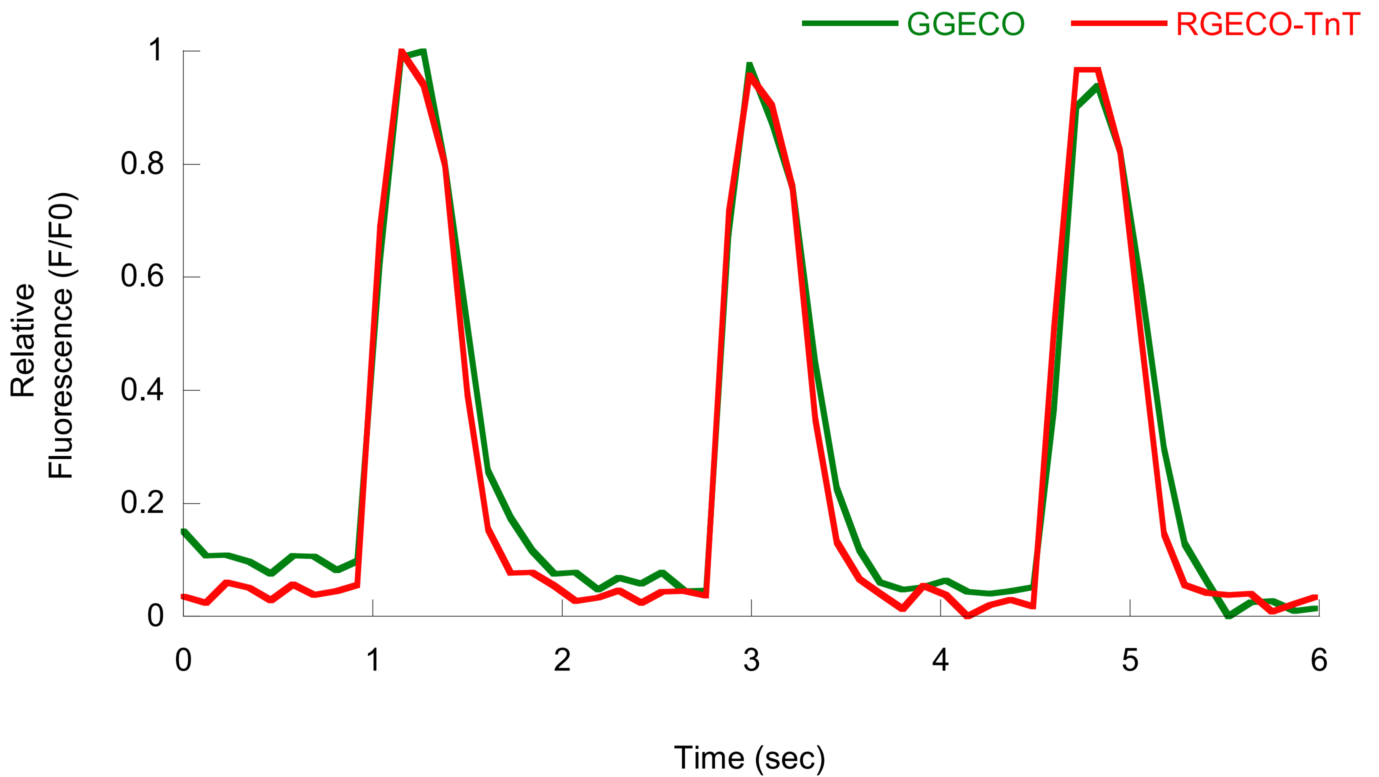
Supplementary Figure 8. Subcellular fractionation of GPCMs expressing RGECO-TnT. The relative subcellular incorporation of RGECO-TnT was assessed by subcellular fractionation of GPCMs 48hours after adenoviral infection. Western blots using anti-cTnT gave a 38kDa endogenous band and a 80kDa conjugate band exclusively on the sarcomeric fraction (sarc) **(a)**. Re-probing with the cytoplasmic marker ERK showed that subcellular fractionation was of high fidelity **(b)**.



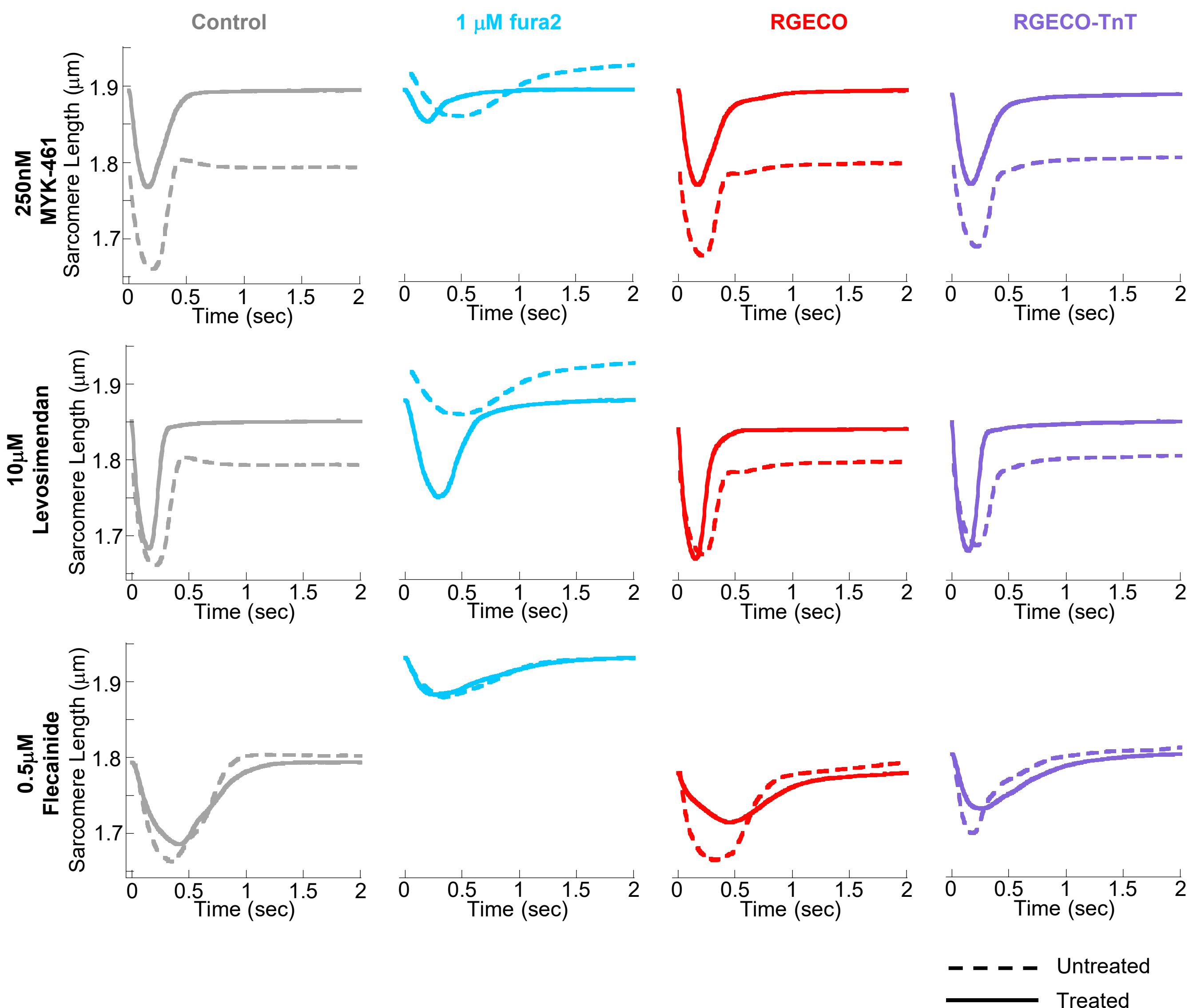
Supplementary Figure 9. RGECO and RGECO-TnT Ca^{++} transients show reverse rate dependence in times to 50% on and 50% off in response to increased pacing frequency in adult GPCMs. Averaged Ca^{++} transients of electrically paced isolated adult cardiomyocytes was used to compare 0.5Hz (black), 1.0Hz (orange) and 2Hz (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 10. 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 0.5Hz (black), 1.0Hz (orange) and 2Hz (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 11. 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 12. 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
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AGGCTGAGACCGAGGAGACCAGGGCAGAAGAAGATGAAGAAGAAGAGGAAGCAAAGGAGGCTGAAGATGGCCCAATGGAG
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TGACATCCACCGGAAGCGCATGGAGAAGGACCTGAATGAGTTGCAGGCGCTGATTGAGGCTCACTTTGAGAACAGGAAGA
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AATGAGCGGGAGAAGGAGCGGCAGAACCGCCTGGCTGAAGAGAGGGCTCGACGAGAGGAGGAGGAGAACAGGAGGAAGGC
TGAGGATGAGGCCCGGAAGAAGAAGGCTTTGTCCAACATGATGCATTTTGGGGGTTACATCCAGAAGCAGGCCCAGACAG
AGCGGAAAAGTGGGAAGAGGCAGACTGAGCGGGAAAAGAAGAAGAAGATTCTGGCTGAGAGGAGGAAGGTGCTGGCCATT
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CGACCTGCAGGAGAAGTTCAAGCAGCAGAAATATGAGATCAATGTTCTCCGAAACAGGATCAACGATAACCAGAAAGTCT
CCAAGACCCGCGGGAAGGCTAAAGTCACCGGGCGCTGGAAATAG

RGECO-TnT amino acid Sequence

MVDSSRRKWNKAGHAVRAIGRLSSPVVSERMYPEDGALKSEIKKGLRLKDGGHYAAEVKTTYKAKKPVQLPGAYIVDIKL
DIVSHNEDYTIVEQCERAEGRHSTGGMDELYKGGTGGSLVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRP
YEAFQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYIKHPADIPDYFKLSFPEGFRWERVMNFEDGGIIHVNQDSSLQDGV
FIYKVKLRGTNFPPDGPVMQKKTMGWEATRDQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDM
INEVDADGDGTFDFPEFLTMMARKMNDTDSEEEIREAFRVFDKDGNGYIGAAELRHVMTDLGEKLTDEEVDEMIRVADID
GDGQVNYEEFVQMMTAKGSMSDIEEVVEEYEEEEQEEAAVEEQEEAAEEDAEAEAETEETRAEEDEEEEEAKEAEDGPME
ESKPKPRSFMPNLVPPKIPDGERVDFDDIHRKRMEKDLNELQALIEAHFENRKKEEEELVSLKDRIERRRAERAEQQRIR
NEREKERQNRLAEERARREEEENRRKAEDEARKKKALSNMMHFGGYIQKQAQTERKSGKRQTEREKKKKILAERRKVLAI
DHLNEDQLREKAKELWQSIYNLEAEKFDLQEKFKQQKYEINVLRNRINDNQKVSKTRGKAKVTGRWK.

Supplementary Figure 13. 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	TnI-GFP
ΔpCa_{50}	-0.031±0.018	-0.149±0.056	0.564±0.269	-0.369±0.125	-0.279±0.624
Δn_H	0.980±0.691	7.308±3.703	0.8649±0.598	-0.867±0.912	**-2.158±0.170 ** **
ΔMin (sec^{-1})	0.548±0.219	0.185±0.089	**6.551±0.969 *** **	**5.382±1.874 * **	**3.096±1.105 * **
ΔMax (sec^{-1})	0.686±0.588	**-2.958±0.399 * **	1.479±0.798	**1.439±0.545 * **	0.787±0.457

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 4**. n=5, significance values comparing unconjugated with GFP conjugated troponin are $p<0.001$=***, $p<0.01$=** and $p<0.05$=*. For ease of interpretation abnormal results are red, only GFP fused to the amino terminus of TnT appears not to disrupt the function of the myosin S1 ATPase.

	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})) (565nm)	32.39	34.53
Molar Extinction coefficent pCa 8.5 (ε (mM^{-1}•cm^{-1})) (565nM)	5.17	6.64
Molar Extinction coefficent pCa 4.5 (ε (mM^{-1}•cm^{-1})) (455nm)	4.16	6.18
Molar Extinction coefficent pCa 8.5 (ε (mM^{-1}•cm^{-1})) (455nM)	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 ($F_{365/380}$)
1μM fura2	DMSO	22	0.282±0.019	0.035±0.005	0.278±0.014	1.091±0.026
	250nM 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	-
	250nM 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	-
	250nM 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
	250nM MYK-461	36	7.177±0.500	1.892±0.007 ***	0.063±0.004	0.102±0.006 *
1μM fura2	DMSO	20	4.351±0.534	1.925±0.014	0.231±0.018	0.395±0.031
	250nM 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
	250nM 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
	250nM 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 250nM MYK-461. Extracted values from paired experimental comparisons of MYK-461 to control in table **(a),** were taken from Ca^{++} transients in **Figure 4a**. **(b)** gives parameters from unloaded sarcomere shortening in **Supplementary Figure 12.** n = total cell number , standard error and significance were taken from RGECO and RGECO-TnT Ca^{++} transients in Figure 4b 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 ($F_{365/380}$)
1μM fura2	DMSO	22	0.286±0.018	0.035±0.005	0.287±0.015	1.105±0.026
	10μM 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	-
	10μM 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	-
	10μM 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
	10μM Levosimendan	36	**9.266±0.409 ****	**1.849±0.010 ****	**0.045±0.002 *****	**0.071±0.003 *****
1μM fura2	DMSO	22	4.392±0.491	1.926±0.015	0.232±0.017	0.386±0.028
	10μM 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
	10μM 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
	10μM 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 10μM Levosimendan. Extracted values from paired experimental comparisons of Levosimendan to control in table **(a),** were taken from Ca^{++} transients in **Figure 4a**. **(b)** gives parameters from unloaded sarcomere shortening in **Supplementary Figure 12.** n = total cell number , standard error and significance were taken from RGECO and RGECO-TnT Ca^{++} transients in Figure 4b 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 ($F_{365/380}$)
1μM fura2	DMSO	*24*	0.257±0.027	0.037±0.004	0.302±0.023	1.086±0.018
	0.5μM 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	-
	0.5μM 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	-
	0.5μM 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
	0.5μM Flecainide	*24*	**6.404±0.354 ****	1.789±0.012	**0.160±0.014 ***	**0.262±0.015 ****
1μM fura2	DMSO	*25*	3.274±0.388	1.932±0.013	0.150±0.015	0.348±0.019
	0.5μM 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
	0.5μM 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
	0.5μM 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 0.5µM Flecainide. Extracted values from paired experimental comparisons of Flecainide to control in table **(a),** were taken from Ca^{++} transients in **Figure 4a**. **(b)** gives parameters from unloaded sarcomere shortening in **Supplementary Figure 12.** 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 $p<0.05$=*.