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Title: Assaying the myosin super relaxed (SRX) state across muscle types, cells, and proteins for understanding muscle biology and use in drug discovery.

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Editorial summary: The Mant-ATP assay is a simple and accessible means for rapid quantitative assessment of the ratio of super-relaxed myosin to disordered relaxed myosin. This protocol provides a standardized methodology to perform the assay across a range of systems.

Proposed teaser: Assaying super relaxed myosin with Mant-ATP

Key Papers:

Toepfer, C.N., et al., Myosin Sequestration Regulates Sarcomere Function, Cardiomyocyte Energetics, and Metabolism, Informing the Pathogenesis of Hypertrophic Cardiomyopathy. Circulation, 2020. 141(10): p. 828-842. https://doi.org/10.1161/CIRCULATIONAHA.119.042339

Toepfer, C.N., et al., Hypertrophic cardiomyopathy mutations in MYBPC3 dysregulate myosin. Sci Transl Med, 2019. 11(476). https://doi.org/10.1126/scitranslmed.aat1199

Gollapudi, S.K., et al., Synthetic thick filaments: A new avenue for better understanding the myosin super-relaxed state in healthy, diseased, and mavacamten-treated cardiac systems. J Biol Chem, 2021. 296: p. 100114. https://doi.org/10.1074/jbc.RA120.016506

Lewis, C.T.A., et al., Remodelling of Skeletal Muscle Myosin Metabolic States in Hibernating Mammals. eLife, 2024. https://doi.org/10.7554/eLife.94616.2

Abstract

The myosin super relaxed state (SRX) is a biochemical and structural conformation of myosin that modulates contractility and energy expenditure and is in equilibrium with the disordered relaxed state of myosin (DRX), which can hydrolyse ATP to produce force. The proportion of myosin SRX:DRX is perturbed in a variety of muscle disorders, and myosin SRX:DRX has become a promising drug target. There are many approaches that can be used to interrogate myosin conformations including X-ray diffraction, stopped flow kinetics, and electron microscopy. These techniques are highly informative but necessitate highly skilled researchers and specialist equipment, limiting wider uptake and accessibility. For this reason, we provide a set of protocols detailing established assays to measure biochemically defined myosin SRX:DRX in skeletal muscle, cardiac muscle, induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs), myofibrils, reconstituted thick filaments, and isolated molecular motors using a simple chase assay incorporating a fluorescent ATP analogue: Mant-ATP. The Mant-ATP assay provides a biochemical measure of myosin states that is distinct from assays that are used to visualise myosin structure directly. These Mant-ATP assays have a variety of protocol lengths, ranging one-two days for preparation and thirty minutes to run an experiment. With this set of protocols, we make the Mant-ATP assay accessible to those working in biochemistry, muscle physiology, and cell biology. At the end of this protocol, users should be able to ascertain a clean fluorescent decay trace that can be fit to define the ratio of SRX:DRX myosin in their sample of choice.

Key points

- We describe a set of Mant-ATP assays for use across tissues, cells, reconstituted filaments, and molecular motors. These assays provide rapid quantitative assessment of the ratio of myosin molecules in the biochemically defined super-relaxed (SRX) and disordered relaxed (DRX) states.

- These Mant-ATP protocols are accessible and use widely available fluorescent microscopy set-ups, providing a simpler method to assay SRX:DRX than high skill X-ray diffraction or electron microscopy studies.

[H1] Introduction

Striated muscle myosins drive locomotion and heart function by forming cross-bridges with actin, going through a chemo-mechanical cycle, hydrolysing ATP, and performing a powerstroke that produces the force necessary to shorten sarcomeres. This process is energy-intensive, necessitating tight homeostatic control to ensure healthy cellular function, which is especially true in the heart [1].

Filamentous myosins can exist in a variety of structural conformations and biochemical states. The first is a structurally varied state of filamentous myosins that are available to bind actin and drive muscle contraction termed disordered relaxed (DRX) myosins, which occupy a variety of structural states and proximities to actin when viewed by X-ray diffraction or electron microscopy [2,3]. Notably, DRX myosins have an ATP turnover rate of ~0.01-0.03s^{-1}. DRX myosins exist in a dynamic equilibrium with super relaxed myosins (SRX), which have a ten-fold lower ATP turnover rate of ~0.001-0.003s^{-1} [4-6]. It is hypothesised that these SRX myosins can fold back towards the backbone of the thick filament becoming sequestered away from actin, reducing cross-bridge cycling and muscle contraction [7].

The SRX state(s) of myosin are modulatory of contractile activity rather than an 'on' and 'off' mechanism like that observed in thick filament regulation of smooth muscle [8], or the calcium-based thin filament regulation of striated muscle [9,10]. Recruitment of cross-bridges is dynamic and influenced by a variety of factors including pre-load, specific post-translational modifications of contractile proteins and their location within the sarcomere [11-15]. Dynamic change in myosin SRX abundance underlies adaptation of cardiac output and has seemingly been adapted in mammalian evolution and used in hibernation [16,17]. This paper presents an approach that can be used to assay SRX:DRX equilibria across a variety of assay types, by employing the fluorescent Mant-ATP probe.

[H2] Development of the protocol

[H3] The Mant-ATP assay

The Mant-ATP assay is based on the single turnover kinetics of a fluorescent ATP analogue called Mant-ATP (2'/3'-O-(N-Methylanthraniloyl) Adenosine 5'-Triphosphate). Mant-ATP binding to myosin increases its fluorescence, and remains high when the myosin in the sample is bound to either Mant-ATP or Mant-ADP, but its fluorescence drops when the Mant nucleotide dissociates from myosin (Figure 1A) [4,5]. This feature of the Mant-nucleotide makes it attractive to separate the association and dissociation events. Mant-ATP can be easily sourced from commercial suppliers, fluoresces brightly, and washes in and out of permeabilised tissue systems (Figure 1B).

Inference of SRX:DRX ratios using Mant-ATP nucleotides is predicated on the observation that DRX myosin binds and un-binds ATP and its analogues an order of magnitude faster than SRX myosin [4]. After steady-state saturation with Mant-ATP, one can use a chase experiment to wash fluorescent Mant-ATP out of the preparation with a non-fluorescent or "dark" ATP solution. The fluorescent decay that is visualised can be fit by a double exponential decay, with the myosins in the DRX state binding and releasing Mant-ATP/ADP faster than those in the SRX state (Figure 2). Thus, the fluorescent decay can be separated into fast and slow washout phases that correspond to DRX and SRX ratios within the muscle preparation.

To quantify the myosin populations in the SRX and DRX states, fluorescence decay data are normalized to background fluorescence and constrained to fluorescence values running from 1 to 0, with maximum fluorescence corresponding to the fluorescence observed at the moment of Mant-

ATP saturation, and minimum fluorescence corresponding to the fluorescence observed at t = fluorescence plateau (Figure 2).

These normalized data are then fitted to a two-phase decay function.

$$Normalised\ Fluorescence = 1 - P_1\left(1 - \exp^{\left(-t/T_1\right)}\right) - P_2\left(1 - \exp^{\left(-t/T_2\right)}\right)$$

The function is used to estimate four parameters: amplitude and the rate parameters for the fast and slow phases, that are later used to define the DRX and SRX states, respectively. P1 corresponds to the amplitude of the initial rapid decay (related to DRX) with T1 as the time constant for this decay. P2 corresponds to the amplitude of the slower second phase of decay (related to SRX) with its associated time constant T2. The amplitude, representing the percentage contribution of the slow phase of fluorescence decay, acts as a proxy for the proportion of myosin heads in the SRX state.

Importantly, only a portion of the fluorescence decay during the rapid phase (P1) corresponds to specifically-bound Mant-ATP being displaced from myosin ATPase sites by unlabelled ATP. Some of the initial fluorescence decay corresponds to the washout of non-specifically bound or unbound Mant-ATP. Therefore, P1 represents an overestimate of DRX myosin, whilst P2 represents an underestimate of SRX myosin. This proportion of fluorescence attributable to non-specifically bound Mant-ATP nucleotides can be quantified through competition with ATP in an initial incubation [23]. Competitive exclusion of Mant-ATP by excess ATP ensures that all of the fluorescence observed in the incubation is from non-specifically bound Mant-ATP. Previous authors have shown that 41% of the initial fluorescence in psoas muscle fibres is non-specific and 52% is non-specific in cardiac fibre bundles [23]. As there is variability between model systems and setups, the fraction of non-specifically bound Mant-ATP should be quantified in each model system independently and the estimated fraction of myosins in the SRX state should be adjusted accordingly (See Additional Procedures: Box 1 for more details). To obtain SRX values, P2 is adjusted to account for the fluorescence decay caused by the washout of non-specifically bound or unbound Mant ATP in the fast phase. The proportion of DRX is then calculated as equal to 1 – SRX.

[H3] Compatible systems

Cells and tissues can be chemically permeabilised, facilitating manipulation of the sarcomeric ATP concentration and removal of ATPases channels. Permeabilised samples can then be mounted within flow chambers for rapid exchange of solutions. Initially, incubation with buffers lacking ATP induces a rigor state. Subsequently, flow chambers can be flushed with Mant-ATP and then “dark” ATP to assess fluorescence uptake and decay.

This approach was first used with rabbit psoas skeletal muscle fibres, helping to first define the myosin SRX state [4]. The same lab then developed the Mant-ATP assay to confirm the presence of SRX myosin in cardiac muscle fibres [5]. Subsequent authors have adapted this work for use in a range of model systems [16,18,19].

The relatively easy implementation of the Mant-ATP assay has caused a significant divergence in methods, data analysis, and results. In this paper, we bring these authors and their methods together to describe a standardized protocol for performing the Mant-ATP assay to assess myosin SRX and DRX ratios in skeletal muscle tissue, cardiac muscle tissue, induced pluripotent stem cell

derived cardiomyocytes (iPSC-CMs), muscle fibres, myofibrils, reconstituted thick filaments, and isolated molecular motors (Figure 1B). We provide detailed instructions for sample preparation, assay conditions, data acquisition, and data analysis, along with troubleshooting tips and potential sources of error. Our aim is to provide a comprehensive and reliable method for researchers to use in their investigations of myosin regulation and function, and to ensure the reproducibility and comparability of Mant-ATP assay data across different laboratories and experimental conditions.

[H2] Comparisons with other methods

Recent advances in the field have highlighted the important structural motifs associated with the SRX state(s) of myosin. This collection of inter and intramolecular interactions are called the interacting head motif (IHM) [20]. Destabilisation of the IHM interactions have been implicated in hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), and Duchene Muscular Dystrophy (DMD) [7,21-23]. These findings have highlighted the importance of the myosin IHM and formation of SRX for long-term health in muscle tissues.

High precision methods such as X-ray diffraction studies and electron microscopy have previously been applied to assess IHM structures, as a proxy to measure SRX proportions[7,24]. These assays provide valuable insight but require specialised equipment and training that are not available to many research groups. Importantly, changes in myosin structure do not necessarily equate to changes in myosin biochemical function, and the opposite is also true. This has been highlighted recently by a study using X-ray diffraction where the myosin activator omecamtiv mecarbil and an SRX-disrupting molecule piperine both move myosin heads away from the thick filament into an 'on' state with no alteration in the proportion of myosin heads in the SRX state(s) [25]. This indicates the necessity for both biochemical and structural assays to quantify myosin conformations and functions, as neither structural nor biochemical assessments alone provide the full picture. This is where new derivations upon the biochemical Mant-ATP assay have come to the fore, by providing an experimentally simple and accessible means for the rapid quantitative appraisal of slow and fast ATP turnover, distinctly separate but complementary to any assessments of myosin structure [5].

[H2] Limitations

The Mant-ATP assay has identified changes in the SRX state due to cardiomyopathy causing mutations in sarcomere proteins, altered myosin regulation by cMyBPC phosphorylation, myosin conformation by pharmacological agents, myosin light chain regulation, and others [11,16,21,26]. Many of these studies were carried out independently with a notable variability in the method and differences in the reported values for defining the SRX population, indicating a need for a standardised protocol.

As previously emphasised, the Mant-ATP assay itself does not evaluate the structural state of myosin and solely investigates the biochemical states of myosin. The equilibrium of these two biochemical states is easily perturbed, such that assay conditions must be carefully controlled to ensure accurate measurements for the different substrates under investigation. Experimental conditions including temperature, ionic strength and protein phosphorylation may influence SRX:DRX proportions,

emphasising the importance of carefully controlling these factors[27]. Treatment with small molecules including mavacamten [28] , blebbistatin [29] and dATP [18,30] have also been known to modulate the SRX:DRX equilibrium, as well as genetic mutations in sarcomeric genes. Mohran and colleagues highlight the importance of stabilising these conditions, as rapid turnover of the SRX state(s) to the DRX state enables the liberation of all Mant-ATP molecules from DRX myosin heads, removing the second phase of fluorescence decay [31]. Control of these experimental conditions known to increase SRX:DRX interconversion should preserve nucleotide turnover from the SRX state and allow data to be best described by a double exponential. The suitability of this fit can be evaluated in PRISM.

It is also necessary to quantify the fraction of fluorescence decay that can be attributed to photobleaching. Previous authors have shown this to be a negligible fraction, but it is important for authors to confirm this with their own equipment setup [5].

[H2] Overview of the procedure

We describe steps required to prepare samples and use them in a stopped flow chamber-based Mant-ATP assay for three cell and tissue-based systems which require chemical permeabilization: cardiac tissue in Procedure 1, single skeletal muscle fibres in Procedure 2 and iPSC-derived cardiomyocytes in Procedure 3. In Procedure 4 we describe how to prepare cardiac myofibrils (Step 1, Option A), cardiac muscle tissue homogenates (Step 1, Option B) and reconstituted myosin filaments (Step 1, Option C), for use in either a stopped flow instrument-based Mant-ATP assay (Step 2, Option A), or a high-throughput, 96-well plate-based Mant-ATP assay. In Procedure 5, we then describe steps to analyse the resulting data to calculate the SRX percentage.

[H2] Experimental Design

[H3] Permeabilised Cardiac Muscle Tissue (Procedure 1)

Permeabilised cardiac tissue has been used as a reliable model system for examining SRX:DRX proportions, initially reported by Hooijman et.al [5]. This technique has been adapted and used to study various myopathy-linked mutations [16,21,23,26,32]. Permeabilised muscle tissue enables SRX dynamics to be studied in the context of all other related sarcomeric proteins and is the most physiologically relevant as the whole tissue is used. Using skinned muscle also means that frozen biopsy tissue can be used for the assay, opening up the ability to use the assay in well-preserved frozen tissue sections.

[H3] Skeletal Muscle Single-Fibre Preparations (Procedure 2)

The first Mant-ATP assay was designed to assess myosin confirmations in skinned rabbit psoas and soleus muscles [4,5]. This approach has been used by a variety of laboratories and was also adapted by the same group to be used in cardiac muscle preparations.

[H3] Induced pluripotent stem cell derived cardiomyocytes (Procedure 3)

iPSC-CMs are an invaluable tool for asking fundamental questions about cardiomyocyte biology. With the challenges of isolating and culturing primary human cardiomyocytes, cardiomyocytes derived en masse from human iPSCs have become an increasingly popular platform for studying human cardiovascular disease[33]. Whilst iPSC-CMs do not fully recapitulate the phenotype of adult cardiomyocytes, they develop sarcomeres, display cardiac-specific gene and protein expression and are capable of spontaneous contraction [34,35]. As such, they can provide insight into SRX:DRX regulation in health and disease [16].

[H3] Myofibrils (Procedure 4, Step 1, Option A)

Myofibrils are the contractile fibers within the myocytes of striated muscle, consisting of sarcomeres linked in series. Importantly, they retain physiological relevance after isolation, as their sarcomeres keep the 3D architecture and protein composition of sarcomeres in their native context. Measuring ~1 µM in diameter, the small size of myofibrils enables rapid diffusion of Mant-ATP into sarcomeres. Furthermore, myofibrils are robust enough to survive rapid mixing in a stopped flow instrument, enabling a high-throughput approach to assessing SRX:DRX population ratios. Myofibrils can be prepared from muscle tissue including fast twitch, slow twitch and cardiac muscle and from iPSC-CMs [18]. They can be used for testing malfunction of thick filament regulation and to explore the potential of small-molecule drug treatments [36].

[H3] Cardiac Muscle Tissue Homogenates (Procedure 4, Step 1, Option B)

Apart from permeabilised tissue, many other physiologically-relevant derivates of muscle tissue, including muscle homogenates have also been employed to study myosin SRX and are convenient when dealing with precious samples available in small amounts (unpublished data).

[H3] Purified Myosin Systems (Procedure 4, Step 1, Option C)

When studying myosin in disease backgrounds that cause different types of myopathies, researchers find it beneficial to work with simpler biochemically reconstituted myosin systems such as S1, heavy meromyosin (HMM), and synthetic thick filaments (STFs). These systems allow investigation of the SRX state(s) of myosin across a variety of myosin complexities [19,28,36,37].

- **S1:** Among these, the shortest and simplest system is the short-S1 (sS1). The sS1 is a single myosin head domain with only the essential light chain (ELC), distinct from S1 which is a single myosin head with both the ELC and regulatory light chain (RLC).
- **HMM:** The HMM system is a double-headed myosin system that includes the proximal part of the myosin tail whose length can vary. Researchers have found that at least the first 15 heptads of the myosin tail domain are required to form the folded-back, IHM-like configuration.
- **STFs:** Finally, STFs are in vitro bipolar arrangements of reconstituted purified full-length myosin that have multiple molecules self-assembled mimicking the native thick filaments. Just like native thick filaments, these reconstituted STFs retain a 14.3-nm myosin subunit periodicity, 43-nm axial periodicity and 3-fold rotational symmetry.

Compared to myosin S1 and HMM, STFs offer an advantage not only to study the underlying biology of thick filaments in a native-like context but they also permit the controlled addition of binding partners such as MyBPC to build a more complex and physiologically relevant system. Indeed, recent studies have employed STFs to understand cooperative myosin activation in thick filaments by perturbing ADP:ATP ratio, RLC phosphorylation, and Ca^{2+} [19,38,39]. These properties can be better studied in STFs that retain many of the inter and intramolecular interactions typically present in the native thick filaments.

The in vitro STF system more closely resembles the biology of the in vivo native thick filaments, which closely mimic the folded-back structure(s) of myosin one would see natively [40-42]. But experimental considerations should be taken regarding the source of purified myosin:

- **Recombinant systems:** These allow researchers to introduce variants of choice that can be expressed as recombinant myosin, allowing the assessment of any disease specific variant of choice. However, the yield of recombinant protein is low in the larger myosin constructs that can be used for STF production, where it is far simpler to produce recombinant S1, which do not capture all of the emergent properties of the IHM.
- **Tissue purification:** When tissue is available for a variant of choice it can be used to extract high yields of full-length myosin. However, tissue availability is often limiting for the study of many variants, especially in the human myosin setting.

We note that STFs may not fully recapitulate the biochemical activity of native thick filaments as the SRX state(s) are likely an emergent phenomenon caused by higher order protein structure within intact thick filaments. Nevertheless, purified myosins and STFs have been shown to serve as useful model systems with myosin populations observed to be in SRX and DRX [19].

[H1] Materials

[H2] Biological Materials

- Human iPSCs Human iPSC lines, such as the KOLF2.1J and PGP1, are commercially available (Jackson Laboratory) or can be derived in-house.

⚠ **CRITICAL** Low passage human iPSCs should ideally be used. For best practice, cell lines should be regularly evaluated for genome stability and pluripotency following continual passaging and/or genome editing. Preferred iPSC culture media may differ between cell lines.

CAUTION The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

- Cardiac Muscle Tissue. These can be obtained from a range of species, most commonly pig, mouse or rabbit.

⚠ **CRITICAL** Cardiac samples must be snap frozen in liquid nitrogen as quickly as possible to preserve the phosphorylation states of cardiac proteins which can alter myosin conformation.

- Skeletal Muscle Tissue. These can be obtained from a range of species, most commonly pig, mouse or rabbit.

⚠ **CRITICAL** Samples obtained from skeletal muscles must be snap frozen in liquid nitrogen quickly to avoid any unwanted effects on post-translational modifications or protein content.

[H2] Reagents

CRITICAL The required reagents for each assay are shown in Table 1.

CAUTION With **all** reagents, wear appropriate personal protective equipment including eye protection, appropriate disposable gloves and a lab coat when handling. If brought into contact with skin, wash

immediately with water. Certain chemicals may need to be handled with care and only used in a chemical fume hood to avoid inhalation.

- Matrigel (Corning, cat. no. 356231)
- Dulbecco's Modified Eagle Medium-F12 (DMEM/F-12; Gibco, cat. no. 11330-032)
- Essential 8 (E8) Medium (Gibco, cat. no. A1517001)
- UltraPure 0.5M EDTA (Thermo, cat. no. 15575020)
- Dulbecco's phosphate-buffered saline (DPBS; Gibco, cat. no. 14190-144)
- Y27632 dihydrochloride (Rho kinase inhibitor, Selleck, cat. no. S1049)
- Dimethyl sulfoxide (Sterile-filtered) (DMSO; Sigma, cat. no. D2650)
- Glucose-free RPMI 1640 medium (Thermo, cat. no. 2187503)
- 10 mL B27 Supplement Minus Insulin (Gibco, cat. no. A1895601)
- 10 mL B27 Supplement with Insulin (Gibco, cat. no. 17504044)
- CHIR99021 (Selleck, cat. no. S2924)
- IWR-1 (Selleck, cat. no. S7086) (or other applicable WNT inhibitors)
- Sodium chloride (NaCl; Merck, cat no. S9888)
- Magnesium chloride ($MgCl_2$; Merck, cat no. M2670)
- Ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA; Merck, cat no. 324626)
- Sodium azide (NaN_3; Fisher Scientific, cat no. 10776301)

⚠ **CAUTION** Sodium azide is considered extremely hazardous. Handle with caution.

- Dipotassium phosphate (K_2HPO_4; Fisher Scientific cat no. 10030340)
- Monopotassium phosphate (KH_2PO_4; Merck, cat no. 1.04877)
- ATP (Adenosine triphosphate; Merck, cat no. A2383)
- 2,3-Butanedione monoxime (BDM; Merck, B0753)
- Dithiothreitol (DTT; Merck, cat no. 3860)

⚠ **CAUTION** DTT is considered hazardous. Avoid breathing in fumes.

- Triton-X 100 (Fisher Scientific cat no. 10591461)
- Potassium acetate (CH_3COOK; Merck, cat no. P1190)
- Magnesium acetate (Merck, cat no. M5661)

⚠ **CAUTION** Magnesium acetate may form combustible dust concentrations in air.

- 3-(N-Morpholino)propanesulfonic acid sodium salt (MOPS; Merck, cat no. M9381)
- Glycerol (Merck, cat no. G7757)
- 2'/3'-O-(N-Methylanthraniloyl) Adenosine 5'-Triphosphate (Mant-ATP; Invitrogen, cat no. m12417)
- Potassium Chloride (KCL; VWR, cat no. PAL4591)
- Ethylenediaminetetraacetic acid (EDTA; VWR, cat no. JT8993-1)
- Phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, cat no. 10837091001)

⚠ **CAUTION** PMSF is considered extremely hazardous.

- Sodium bicarbonate ($NaHCO_3$; VWR, cat no. JT3509-1)
- K-pipes (Sigma-Aldrich, cat no. P6757-1kg)
- Tris hydrochloride (Tris-HCL; Sigma-Aldrich, cat no. 648317-1kg)
- Imidazole (Sigma-Aldrich, cat no. I2399)
- BES (Goldbio, cat no. 10191-18-1)
- K-propionate (Sigma Aldrich, cat no. 13112-100G)
- Adenosine 5´-triphosphate disodium salt (Na_2ATP; cat no. 01897-1G)
- Leupeptin (Sigma-Aldrich, cat no. L2884-25mg)

- Pepstatin (Fisher, cat no. AAJ60237MB)
- Adenosine 5′-triphosphate magnesium salt (ATP-Mg; Sigma-Aldrich cat no. A9187)
- Magnesium (Mg, Sigma-Aldrich, cat no. 13112-100G)
- Calcium (Ca, Sigma-Aldrich, cat no. 215147-5G)
- Creatine phosphate (Sigma-Aldrich, cat no. 10621714001)
- Potassium Hydroxide (Fisher Scientific, cat no. P250-500)

⚠ **CAUTION** Potassium hydroxide can cause severe burns.

- TCEP (ThermoFisher Scientific, cat no. J60316.09)
- Trypsin (Sigma-Aldrich, cat no. T0303-1G)
- Trypsin inhibitor (Fisher Scientific, cat no. NC9065058)
- Sucrose (Fisher Scientific, cat no. S5-3)
- Sylgard 184 Silicone Elastomer

Table 1: Required reagents for each assay.

	iPSC-CM	Permeabilised Muscle Tissue	Synthetic Thick Filaments	Myofibril	Cardiac Muscle Tissue Homogenates	Single Fibres
NaCl	✓	✓	✓			
MgCl2	✓	✓	✓	✓	✓	
EGTA	✓	✓	✓	✓	✓	✓
NaN3	✓	✓		✓	✓	
K2HPO4	✓	✓				✓
KH2PO4	✓	✓	✓			✓
ATP	✓	✓	✓	✓	✓	✓
BDM	✓	✓				
DTT	✓	✓	✓			
Triton-X 100	✓	✓				
K acetate	✓	✓				
Mg acetate	✓	✓				✓
MOPS	✓	✓				✓
Glycerol	✓	✓				✓
Mant-ATP	✓	✓	✓	✓	✓	✓
KCL			✓	✓	✓	✓
EDTA			✓			
PMSF			✓		✓	
NaHCO3			✓			
K-pipes			✓			
Tris-HCL			✓	✓	✓	
Imidazole				✓		✓
BES					✓	
K-propionate					✓	
Na_2ATP					✓	
Leupeptin					✓	
Pepstatin					✓	
DMSO			✓	✓	✓	

Mg-ATP						✓
free Mg2+						✓
free Ca2+						✓
Creatine phosphate		✓				✓
Creatine kinase						
KOH						✓
TCEP						✓
Trypsin			✓			
Trypsin inhibitor			✓			
Sucrose					✓	
Silicone Elastomer		✓				

[H2] EQUIPMENT

[H3] iPSC Culture

- CO_2 humidified incubator
- Laminar Flow Hood
- Liquid Nitrogen Cell Storage Tank
- Water Bath, 37°C
- Cell Counter (e.g. Countess 3 Automated Cell Counter)
- Pipette Controller (e.g. Pipetboy)
- 6-well tissue culture-treated plates
- Pipettes (P10, P20, P200, P1000) (e.g. Pipetman)
- Serologic tip pipettes (5 mL, 10 mL, 50 mL)
- Filter Tips (10 µL, 20 µL, 200 µL, 1000 µL)
- 15 mL and 50 mL conical centrifuge Tubes
- Cell Scraper (e.g. Mini cell scraper biotium 2203)
- Forceps

[H3] Cardiac Tissue Harvesting

- Surgical scalpel
- Fine forceps
- Scissors
- Dissection Microscope
- Petri dish
- Insect pins

[H3] Cardiac Tissue/iPSC-CM Flow Chambers

- Epredia™ SuperFrost Plus™ Adhesion slides (Fisherscientific, cat no. 10149870)
- Insect pins

- Double-sided tape
- Araldite 2-part epoxy adhesive
- Glass Square Coverslips (20mm x 20mm)

[H3] Cardiac Myofibrils

- OmniMixer homogenizer (Cole-Parmer, cat no.WZ-04728-05)
- 1000 uM Polypropylene mesh sheet
- 100 µM mesh filters
- 300 µM mesh filters

[H3] Cardiac Muscle Tissue Homogenates

- OmniMixer Homogenizer (Cole-Parmer, cat no.WZ-04728-05)
- 0.5-inch probe (Thomas Scientific)

[H3] Single Skeletal Muscle Fibres

- 6cm petri dishes
- Academy Microscope Slides (Philip Harris, cat no. B8A76260)
- Menzel Microscope Coverslips (Fisher Scientific, cat no. 11961988)
- TEM Copper Grids (200 mesh x 125 µm pitch) SPI G100 2010C-XA, width, 3 mm)
- Ultra-thinned tweezers
- Superglue
- Dissection Microscope

[H3] Purified Myosin

- Meat Grinder (kitchen style electric for mincing extracted muscle)
- Centrifuge with JS-4.2 bucket rotor and compatible centrifuge bottles or tubes
- Glass Dounce Homogenizer
- Mira Cloth
- Micro-fluidizer with 0.5-2'' probe
- Sorvall GSA centrifuge (or similar) and compatible 250 ml centrifuge bottles

[H3] Flow chamber-based fluorescence acquisition

- Fluorescence Microscope (e.g. Zeiss Axio Scope A1 microscope, Zeiss Axio Observer 3 fluorescent microscope or Olympus IX81 fluorescent microscope)
- 20x air objective lens (e.g. Plan-Apochromat 20x/0.8 objective) or 10x objective lens (Olympus UPlanFLN)
- Standard DAPI filter set
- High sensitivity camera (e.g. Photometrics CoolSNAP HQ2, Axiocam 705 mono camera, Zeiss AxioCam ICm 1 camera, Andor iXon Ultra 897 EMCCD camera)
- 1.5 mL all-black microcentrifuge tubes
- 1.5 mL microcentrifuge tubes (e.g. Eppendorf)

[H3] Stopped Flow Instrument fluorescence acquisition

- Double mixing stopped-flow instrument (e.g. BioLogic [43]
- 5 mL or 10 ml plastic syringes (e.g. Thermo Scientific, cat no.03-377-23)

[H3] Plate-based fluorescence acquisition

- 96-well microplate (e.g. Greiner polypropylene microplate)
- Plate reader (e.g. Tecan Infinite M200 PRO instrument)

[H3] Software

- Imaging software (e.g. cellSens https://www.olympus-lifescience.com/en/software/cellsens/))
- GraphPad Prism v10.2.1 (https://www.graphpad.com/scientific-software/prism/)
- FIJI, ImageJ software (https://fiji.sc/) [44]

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[H2] Reagent Setup

[H3] iPSC Culture and Differentiation

- **Matrigel solution:** Pre-chill pipette tip by pipetting up and down ice-cold DMEM/F12. Prepare Matrigel solution by adding 400 µL Matrigel to 40 mL ice-cold DMEM/F12 (1:100 dilution). Aliquot 7.6 mL into 15 mL conical centrifuge tubes. Keep at -20 °C for long-term storage (6-12 months).
- **E8 medium:** Add 10 mL Essential 8 Supplement (50×) to 490 mL of E8 basal medium. Aliquot 40 mL into 50 mL conical centrifuge tubes. Store the aliquots at 2-8°C for up to 2 weeks or at -20 °C for long-term storage.
- **ROCK-inhibitor, 10 mM:** Prepare 10 mM Y27632 dihydrochloride rho kinase inhibitor dissolved in DMSO. Store stocks at −20 °C for up to 6 months.
- **EDTA/PBS, 0.5mM:** Add 500 µL 0.5M EDTA to 500 mL PBS. Store at room temperature (19-23°C) for up to 6 months.
- **RPMI/B27 minus insulin medium:** Add 10 mL B27 supplement (without insulin) to 500 mL RPMI 1640 medium (with glucose). Aliquot 40 mL into 50 mL conical centrifuge tubes. Store at 2-8°C for up to 2 weeks or at -20°C for up to 6 months.
- **RPMI/B27 with insulin medium:** Add 10 mL B27 supplement (with insulin) to 500 mL RPMI 1640 medium (with glucose). Aliquot 40 mL into 50 mL conical centrifuge tubes. Store at 2-8°C for up to 2 weeks or at -20°C for up to 6 months.
- **RPMI/B27 with insulin medium without glucose:** Add 10 mL B27 supplement (with insulin) to 500 mL RPMI 1640 medium (without glucose). Aliquot 40 mL into 50 mL conical centrifuge tubes. Store at 2-8°C for up to 2 weeks or at -20°C for up to 6 months.
- **CHIR99021 stock, 10 mM**: Prepare 10 mM CHIR99021 stocks dissolved in DMSO. Store stocks at −20 °C for up to 6 months.

- **IWR1 stock, 10 mM**: Prepare 10 mM IWR1 stocks dissolved in DMSO. Store stocks at −20 °C for up to 6 months.

[H3] Sample Preparation: Cardiac Muscle Tissue & iPSC-CMs

- **Dissection solution:** Final Concentrations: 10 mM EGTA, 5.6 mM $MgCl_2$, 100 mM KCL, 20 mM Imidazole, 5mM ATP, 10 mM CrP, 50% Glycerol (v/v), protease inhibitors. Prepare fresh, as cannot be stored.
- **Skinning buffer:** Final Concentrations: 100 mM NaCl; 8 mM $MgCl_2$; 5 mM EGTA; 3 mM NaN_3; 5 mM K_2HPO_4; 5 mM KH_2PO_4; 5 mM ATP; 20 mM BDM; 1 mM DTT; 0.1 % (v/v) Triton-X 100. Bring pH to 6.8, aliquot and store at -20°C for up to 12 months.
- **Glycerinating Solution:** Final Concentrations: 120 mM K acetate; 5 mM Mg acetate; 2.5 mM K_2HPO_4; 2.5 mM KH_2PO_4; 50 mM MOPS, 5 mM ATP; 20 mM BDM; 2 mM DTT; Glycerol, 50 % (v/v). Bring pH to 6.8 at room temperature. Store at -20 °C for up to 12 months.

[H3] Sample Preparation: Single Fibres

- **Relaxing Solution:** Final Concentrations: 5.3 mM ATP, 1 mM $MgCl_2$, 100 mM free KCl, 10 mM imidazole, 2 mM EGTA, 14.5 mM creatine phosphate, 1M KOH for adjustment of ionic strength to 180 mM and pH to 7.0, aliquot and store at -20°C for up to 12 months.
- **Skinning Buffer:** 1:1 mix of glycerol and relaxing solution, can be stored at -20°C for up to 12 months.

[H3] Sample Preparation: Cardiac Myofibrils

- **Myofibril Relaxing Buffer:** Final Concentrations: 10 mM Imidazole (pH 7.2), 75 mM KCl; 2 mM $MgCl_2$; 2 mM EGTA; 1 mM NaN_3. aliquot and store at -20°C for up to 12 months.

[H3] Sample Preparation: Cardiac Muscle Tissue Homogenates

- **Muscle Homogenization Buffer**: Final Concentrations: 50 mM BES, 41.89 mM K-propionate, 5 mM NaN_3, 10 mM EGTA, 6.57 mM $MgCl_2$, 6.22 mM Na_2ATP, 1 µM Leupeptin, 2.5 µM Pepstatin, 50 µM PMSF. Make fresh, as cannot be stored.

[H3] Sample Preparation: Synthetic Thick Filaments

- **Myosin Extraction Buffer:** Final Concentrations: 0.3 M KCL; 0.15 M KH_2PO_4 (pH 6.5); 5 mM MgCl2; 20 mM EDTA; 1 mM ATP; 0.2 mM PMSF. Make fresh.
- **High Salt Buffer:** Final Concentrations: 1.2 M KCl; 0.06 M KH_2PO_4 (pH 6.5); 2 mM $MgCl_2$; 25 mM EDTA; 1 mM DTT. Make fresh.
- **BED Buffer:** Final Concentrations: 0.1 mM $NaHCO_3$; 0.1 mM EGTA; 1 mM DTT. Make fresh.

- **Myosin Storage Buffer:** Final Concentrations: 0.6 M KCl; 20 mM K-pipes (pH 6.8); 1 mM $MgCl_2$; 2 mM $NaHCO_3$; 1 mM EGTA; 1 mM DTT. Make fresh.
- **Full-Length Myosin (FLM) Digestion:** Final Concentrations: 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM DTT, 10 mM $MgCl_2$. Make fresh.
- **Thick Filament Buffer:** Final Concentrations: 20 mM Tris-HCl (pH 7.4), 0 mM KCl, 1 mM EGTA, 3 mM $MgCl_2$, 1 mM DTT. Make fresh.

[H3] Mant-ATP Assay: Cardiac Muscle Tissue & iPSC-CMs

- **Rigor buffer:** Final Concentrations: 120 mM K acetate; 5 mM Mg acetate; 2.5 mM K_2HPO_4; 2.5 mM KH_2PO_4; 50 mM MOPS; 2 mM DTT. Bring pH to 6.8 and store at -20 °C.
- **mATP buffer:** Add 250 µM mATP to rigor buffer in a 1.5 mL all-black microcentrifuge tube. Prepare fresh on the day of use. Final Concentrations: 120 mM K acetate; 5 mM Mg acetate; 2.5 mM K_2HPO_4; 2.5 mM KH_2PO_4; 50 mM MOPS; 2 mM DTT, 250 µM mATP.
- **ATP chase buffer:** Final Concentrations: 120 mM K acetate; 5 mM Mg acetate; 2.5 mM K_2HPO_4; 2.5 mM KH_2PO_4; 50 mM MOPS; 2 mM DTT, 4mM ATP. Bring pH to 6.8 and store at -20 °C.

[H3] Mant-ATP Assay: Single Fibres

- **Common Buffer:** Final Concentrations: 120 mM K-acetate, 5mM Mg-acetate (tetrahydrate), 2.5mM K_2HPO_4, 2.5mM KH_2PO_4, 50mM MOPS, 0.5M EGTA, Adjust pH to 6.8 with 10M KOH. It is stable at -20 °C for at least 6 months, when stored in 50 mL batches.
- **Rigor Buffer:** Add 1 mM tris(2-carboxyethylphosphine) to common buffer. Can be stored at -20°C for 12 months.
- **Mant-ATP Buffer:** Add 250 µM Mant-ATP to rigor buffer in a 1.5 mL all black microcentrifuge tube. Prepare fresh on the day of use and store on ice in the dark until ready for experiments.
- **ATP chase Buffer:** Add 4 mM ATP to rigor buffer in a 1.5 microcentrifuge tube and keep as the Mant-ATP buffer above.

[H3] Mant-ATP Assay: Synthetic Thick Filaments

- Ammonium Sulfate solid
- **SRX Experimental Buffer for Stopped-flow Assay:** Final Concentrations: 20 mM Tris-HCl (pH 7.4), 30 mM KCl (or K Acetate), 1 mM EGTA, 3 mM $MgCl_2$, and 1 mM DTT. Make fresh.

[H3] Mant-ATP Assay: Synthetic Thick Filaments, Myofibrils & Cardiac Muscle Homogenates

- **SRX Experimental Buffer for Plate-based Assay**: Final Concentrations: 10 mM Tris (pH 7.5), 30 mM K Acetate, 1 mM EGTA, 4 mM $MgCl_2$, and 1 mM DTT. Make fresh.
- **PM12 Buffer for making HMM and S1 from full-length Myosin:** 12 mM K-PIPES, pH 6.80, 2 mM $MgCl_2$, 1 mM DTT. Make Fresh.
- **For full-length myosin digests for HMM use:** PIPES/KCl Buffer 50 mM K-PIPES, pH 6.80 at room temperature, 500 mM KCl, 2 mM $MgCl_2$, 1 mM DTT. Made fresh. **For S1 myosin preparations use:**

PIPES/EDTA Buffer 20 mM K-PIPES, pH 6.80 at room temperature, 10 mM EDTA, 1 mM DTT. Make fresh.

- **Low Salt Buffer for HMM preparation:** 10 mM K-PIPES, pH 6.80 at 4 ℃, 20 mM KCl, 1 mM DTT, make fresh.
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[H2] Equipment Setup

[H3] iPSC-CMs

Matrigel-coated six-well plates

- Add 1.2 mL of the Matrigel solution to cover the bottom of the wells of a 6-well plate and incubate at 37℃ for at least 1 hour, or overnight at 4℃. Aspirate the Matrigel solution before addition of the medium. If not used immediately, keep for up to 1 week at 4℃. Do not allow to dry out.

Flow Chambers (See Figure 3b)

1. Mix the two parts of araldite adhesive thoroughly.

2. With a pipette tip, place three small dots of araldite adhesive in a row on the microscope slide near the centre of the slide, towards one edge.

3. Place three insect pins in the adhesive pointing towards the centre of the slide, with their tips converging. Leave to dry.

4. Stacks of double-sided tape define two borders of the chamber and provide a surface for the cover slip to adhere to. Fold double-sided tape over on itself once to create a low stack. Add tape either side of the pins. Trim the edges of the tape.

CRITICAL After completion of the assay, the slides, adhesive and pins can be reused, but the tape must be replaced and the slide thoroughly cleaned.

[H3] Permeabilised Cardiac Tissue

Silicone Elastomer Dish

- Mix two parts of silicone elastomer and pour into a petri dish. Leave to set in a smooth layer at room temperature (can take several hours).

Flow Chambers (See Figure 3a)

1. Mix the two parts of araldite adhesive thoroughly.

2. With a pipette tip, place two small dots of araldite adhesive on either side of the microscope slide near the centre of the slide (see the Troubleshooting guide).

! TROUBLESHOOTING

3. Place two insect pins in the adhesive pointing towards the centre of the slide, with their tips nearly touching. Leave to dry.

4. Stacks of double-sided tape define two borders of the chamber and provide a surface for the cover slip to adhere to. Fold double-sided tape over on itself 5 times to create a low stack. Add tape either side of the pins. Trim the edges of the tape.

CRITICAL After completion of the assay, the slides, adhesive and pins can be reused, but the tape must be replaced and the slide thoroughly cleaned.

[H3] Single Fibres

Flow Chambers (Figure 4)

1. Using a scalpel cut out the middle band of copper on the grid and dispose of it.

2. Glue copper grids onto the microscope slides. The two halves are positioned across from each other realigning all the loose ends and barely touching.

3. Mount three thinly cut strips of double-sided tape on top of each other and arrange to flank each half of the copper grid. Air bubbles in the layers should be avoided.

[H1] Procedure 1- Cardiac Tissue

Timing 2 days

[H2] Part I: Sample Preparation

[H3] Harvesting Cardiac Tissue

Timing 30 minute

⚠ CRITICAL We recommend dissecting prior to freezing to avoid exposing the tissue to multiple freeze-thaw cycles.

⚠ CRITICAL Perform dissection on ice with ice-cold dissection solution.

1. Add 10 mL of ice-cold dissection solution to the petri dish containing the pre-set silicone elastomer.

2. Mount heart in dish using insect pins through heart apex.

3. Using fine forceps and scissors, cut away the atrial tissue and vasculature under a dissection microscope.

4. Make incisions from the top of the heart to open the left and right ventricles.

5. Pin the wall tissue down.

6. Wash away the excess congealed blood by stirring the dissection solution. Ensure all blood clots are removed from the chambers. Replace with fresh ice-cold dissection solution.

7. Dissect tissue to smaller 20 mg pieces.

8. Dry the separated tissue chunks on blotting paper and rapidly snap freeze in liquid nitrogen.

PAUSEPOINT Any tissue sections can then be stored at -80 ^{0}C until they are needed for experimentation.

Permeabilising Cardiac Tissue

Timing 7 hours

9. If not previously stored in small 20 mg chunks, then cut pieces of 20 mg cryopreserved cardiac tissue per sample with a surgical scalpel.

10. Thaw and permeabilize samples using 1 mL skinning buffer in an Eppendorf tube and incubate tubes on ice on a rocker for 6 h. Replace solution with fresh skinning buffer every 120 min (2h skinning buffer – replace skinning buffer – 2 h skinning buffer – replace skinning buffer – 2h skinning buffer).

11. After 6 h, replace skinning buffer with glycerinating solution.

⏸ **PAUSE POINT** Store samples in glycerinating solution on ice on a rocker overnight or at –20 °C.

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Mounting permeabilised tissue on slides

Timing 40 mins per slide

12. Cut a ~ 1mm x 1mm piece of sample using a surgical scalpel and place it in a drop of glycerinating solution between the two stripes of double-sided tape on the flow chamber slide under the two free ends of the pins (Figure 5).

13. Cover the flow chamber by placing a cover slip on top of the sample and attached to the two stripes of double-sided tape.

14. Permeabilize sample for a further 30 minutes by infusing skinning buffer into the chamber. Incubate slide on ice.

15. Replace skinning buffer in flow chamber with glycerinating buffer.

Part 2: Stopped Flow Chamber-Based Chase Assay

Timing 30 min per slide

⚠ **CRITICAL** All solutions should be brought to room temperature, whilst samples in flow chambers must be kept on ice.

⚠ **CRITICAL** This assay must be performed in complete darkness. Avoid exposing the cells to blue light.

⚠ **CRITICAL** For fluorescence acquisition, use a fluorescence microscope with a 10x air objective lens and camera. Acquire frames every five seconds with a 20 ms acquisition/exposure time with 385nm using a standard DAPI filter set, for ten minutes.

16. Prior to fluorescence acquisition, replace glycerinating buffer in the flow chamber by pipetting rigor buffer into the chamber and incubate at room temperature for 5 minutes (Figure 6)

17. Repeat step 16 for another 5 minutes to allow rigor to set in.

18. Place slide on microscope stage and at 10X magnification, use a pin as a reference to focus on the sample. Ensure that two thirds of the field of view consists of the sample whilst one third is the background.

19. Recording should begin prior to chamber infusion, with videos acquired at 5 frames per second. Press record and immediately begin slowly infusing one chamber volume of mATP buffer. Ensure you do not move or dislodge the sample when infusing, keeping the sample in a fixed position. Collect expelled buffers with a tissue pressed up against the end of the flow chamber to facilitate solution exchange by wicking/ capillary action.

20. To flush out the fluorescent mATP buffer, infuse ATP chase buffer and again acquire frames every 5 seconds for 10 minutes. During these 10 minutes, we recommend performing step 16 on the next sample to maximise time efficiency.

21. Fit the normalized fluorescence decay traces to a two-phase decay function to yield the amplitudes and rates of fast and slow phases. See Data Analysis Section (Procedure 5) for more details.

Procedure 2- Single Skeletal Muscle Fibres

Part 1: Sample Preparation

Dissecting Single Skeletal Muscle Fibres

Timing ~3 days

1. In a cryo-chamber set to -20 ^{0}C, cut a 10mg piece of skeletal muscle, from a snap-frozen biopsy, in the longitudinal direction and place it in skinning buffer. (50% Glycerol and relaxing solution).

2. Skin the samples by first keeping them in skinning buffer at -20°C for 24 hours.

3. After 24 hours, move the samples to a fridge set to 5°C for another 24 hours.

PAUSE POINT The now-skinned muscles can be stored at -20°C for up to 1 week.

4. To isolate single fibres, transfer bundles to 6 cm Petri dishes containing a relaxing solution, placed on a dissection microscope (Figure 7).

5. Dissect the bundles into single fibres with ultra-thinned tweezers.

CRITICAL Only the tips of each fibre can reliably be touched, and stretching of fibres must be avoided to preserve sarcomere integrity.

Mounting tissue on slides

6. To mount the sample in the flow chamber, use the open square edges to pin down the ends of each fibre (Figure 8). Up to 6 fibres can reliably be mounted on each grid. Fewer fibres may be mounted depending on fibre size.

CRITICAL STEP Skeletal muscle fibre thickness varies among vertebrate species. To avoid overlapping of Gaussian functions during fluorescent decay, and misinterpretations of DRX/SRX conformations, fibres should be mounted with one free pin in-between each of them. The three centre pins are to be left free and used later for background sampling.

7. Gently attach a 22x22 mm coverslip on top.

Part 2 Stopped Flow Chamber-Based Chase Assay

Timing 30 min per sample

CRITICAL Experiments are carried out at 25°C.

CRITICAL For fluorescence acquisition, our preferred setup is a Zeiss Axio Scope A1 microscope with a Plan-Apochromat 20x/0.8 objective lens and the Zeiss AxioCam ICm 1 camera. Acquire frames every five seconds with a 20 ms acquisition/exposure time at 385nm using a standard DAPI filter set, for a total of five minutes.

8. To exclude damaged fibres, use Brightfield 63x zoom to measure sarcomere length, mark an area, and calculate the average sarcomere length using FIJI 1.54f. Fibres with a sarcomere length of at least ~2.50 µm can be used for the Mant-ATP chase protocol.

9. Wash the flow chamber 3 times for 5 minutes with 100 µL rigor buffer. To add the buffer, use a P100 pipette positioned at an angle against the slide and coverslip and tissue paper on the other side, used to create suction.

10. Now exchange and incubate with 250 µM Mant-ATP in rigor buffer for an additional 5 minutes.

11. Position the prepared flow chamber on the microscope stage, so that one-half of the grid contains three fibres and the free centre pins are visible. The aim is to see one-half of the grid with suspended fibres. For this, you can briefly use Brightfield. (Figure 9).

12. Start the time-lapse. Wait out the two first snaps and use these as zero images for reference. On the third snap, exchange solution in the chamber with ATP-Chase buffer containing 4 mM unlabelled ATP.

⚠ **CRITICAL** Avoid movement of the slide at the time of pipetting.

13. Repeat steps 9 to 12 for the other half of the grid. See data analysis section for downstream analysis (Procedure 5).

Procedure 3- iPSC-CMs

Timing 4-6 weeks

Part 1: Preparing iPSC-CMs

iPSC Culture

Timing 1 week

⚠ **CRITICAL** Standard rules of aseptic cell culture must be rigorously observed. Standard cell culture laminar flow hoods will be required. All items must be sprayed with 70% ethanol before entering the hood. This protocol is antibiotic-free.

1 Thaw a vial of frozen iPSCs in a 37°C water bath.

2 Add 1 mL E8 medium with 10 µM ROCK inhibitor to the vial and transfer iPSCs to a 15 mL conical tube. Add 9 mL E8 medium with 10 µM ROCK inhibitor.

3 Centrifuge for 3 minutes at 200g at room temperature.

4 Carefully aspirate the supernatant. Do not disturb the pellet.

5 Resuspend in 1 mL E8 medium with 10 µM ROCK inhibitor. Perform cell counting.

6 Add 2 mL E8 medium with 10 µM ROCK inhibitor to each well of a Matrigel-coated six-well plate.

7 Seed 100,000 cells per well. Gently shake the plate to evenly distribute cells throughout the wells. Culture the cells at 37°C in 5% CO_2.

8 Check the cells on the day after plating. Cell death is to be expected, but many cells should have adhered to the culture plate and will display a characteristic spikey morphology.

9 At 24 hours after plating, replace the E8 medium with 2 mL fresh E8 medium **without** ROCK inhibitor. Continue to do this daily until cells reach 70-80% confluency, at which point they should be passaged.

⚠ **CRITICAL STEP** Ensure cells do not become overconfluent as pluripotency and future differentiations may be negatively affected. At >70% confluency, iPSCs are beginning to transition out of a logarithmic growth phase and are ready to be passaged.

⏸ **PAUSE POINT** If desired, iPSCs can be cryopreserved at this point, prior to cardiomyocyte differentiation

10 To passage, aspirate E8 medium and add 1 mL 0.5 mM EDTA/PBS per well. Incubate at 37°C for 5-7 minutes to dissociate cells. Observe cells under a microscope, looking for individual colony edges as a sign of dissociation.

11 Gently aspirate the 1 mL of 0.5 mM EDTA/PBS. With a P1000 pipette, use 1 mL fresh E8 medium to rinse the well 7-8 times and dislodge the iPSCs. Collect the cell suspension in a 15 mL conical tube.

12 Add 11 mL fresh E8 medium supplemented with 10 µM ROCK inhibitor to the conical tube to obtain a 1:12 cell dilution. Gently mix the cell suspension.

13 Add 2 mL of the 1:12 cell dilution to 5 wells of a new Matrigel-coated 6 well plate for a 1:6 split. Add 1 mL of the 1:12 cell dilution to the final well, and top up with 1 mL fresh E8 medium with 10 µM ROCK inhibitor **without** cells for a 1:12 split. This format is preferred to provide 5 wells for differentiations and one well for continued passaging.

14 Gently agitate the plate to evenly distribute cells throughout the wells in the 37°C incubator.

15 On the following day, replace the E8 medium with 2 mL fresh E8 medium daily **without** ROCK inhibitor.

iPSC-CM Differentiation

Timing 30 days

CRITICAL Differentiation of iPSCs into cardiomyocytes can be achieved using a 30-day small molecule-mediated protocol centred around temporal modulation of Wnt signalling. Performing this protocol in a 6-well tissue culture plate format forms a beating monolayer of iPSC-CMs appropriate for the Mant-ATP assay. Cells are cultured at 37°C with 5% CO_2.

⚠ **CRITICAL STEP** Warm media to room temperature prior to media changes.

⚠ **CRITICAL STEP** Between days 0-7, media changes must be performed at the same time each day.

16 Initiate differentiations on 5 wells of iPSCs at 90-95% confluency. On Day 0, remove E8 medium. Add 2 mL RPMI/B27 minus insulin medium containing 10 µM CHIR99021 to each well.

! TROUBLESHOOTING

17 On Day 1, add 2 mL RPMI/B27 minus insulin medium to each well, topping up to 4 mL total and diluting the CHIR99021 concentration by half.

18 On Day 2, aspirate and replace with 2 mL RPMI/B27 minus insulin medium without CHIR99021.

19 On Day 3, aspirate and replace with 2 mL RPMI/B27 minus insulin medium containing 5 µM IWR1. Incubate at 37 ℃ and 5% CO2 in this medium 48 hours.

20 On Day 5, aspirate and replace with 2 mL RPMI/B27 minus insulin medium, removing IWR1. Leave for 48 hours.

21 On Day 7, aspirate RPMI/B27 minus insulin medium and replace with 2 mL RPMI/B27 plus insulin medium.

22 Continue to replace with 2 mL fresh RPMI/B27 plus insulin medium every 48 hours.

23 Cells will spontaneously begin beating normally between days 8-10. When beating starts, replace medium with 2 mL RPMI/B27 plus insulin medium without glucose to begin metabolic purification of iPSC-CMs. Replace after 48 hours. Continue with metabolic purification for 4 days.

⚠ **CRITICAL STEP** Cell death is to be expected and cells may slow or stop beating during metabolic purification. If beating is not observed by day 14, the differentiation attempt has likely failed.

24 After 4 days of metabolic purification, aspirate and return cells to 2 mL RPMI/B27 plus insulin medium with glucose.

25 Continue to replace with 2 mL fresh RPMI/B27 plus insulin with glucose every 48 hours.

26 Observe cells on day 28-32; if iPSC-CMs continue to beat as a sheet, they are ready to be harvested for the Mant-ATP assay.

Harvesting iPSC-CMs

Timing ~40 min per sample

⚠ **CRITICAL** 4-6 samples can be harvested per well of a 6 well plate, but only consistent beating sheets should be used.

⚠ **CRITICAL** The sheet of iPSC-CMs is incredibly thin and delicate. Extra care should be taken during dissection and chamber infusions not to disturb or damage the sheet.

27 Place a drop of glycerinating solution over the pins on the flow chamber slide (Figure 10).

28 Dissect a small area (~10mm x 10mm) of beating iPSC-CMs in media using the cell scraper. This can be performed under a microscope or by eye. Dissect and mount samples from one well at a time.

29 Using the forceps, transfer the sample onto the slide. Carefully unfold the sample into a sheet once on the slide.

30 Under the microscope, place the three pins over the sheet using the forceps (Figure 10).

31 Cover the flow chamber by placing a cover slip on top of the sample, attached to the two stripes of double-sided tape. Place the coverslip against one edge of the slide, opposite to the dots of araldite adhesive.

32 Immediately after addition of the coverslip, infuse the chamber with skinning buffer, ensuring the sample is fully covered. Incubate for 30 minutes on ice.

33 Infuse the chamber with glycerinating solution and use immediately.

⏸ PAUSE POINT If desired, the iPSC-CM sample mounted in the flow chamber can be stored at -20°C for up to 3 days.

Part 2: Stopped Flow Chamber-Based Chase Assay

Timing 30 min per sample

⚠ CRITICAL All solutions should be brought to room temperature, whilst samples in flow chambers must be kept on ice.

⚠ CRITICAL This assay must be performed in complete darkness. Avoid exposing the cells to blue light.

⚠ CRITICAL For fluorescence acquisition, use a fluorescence microscope with a 10x air objective lens and camera. Acquire frames every five seconds with a 20 ms acquisition/exposure time with 385nm using a standard DAPI filter set, for ten minutes.

34 Perform Mant-ATP flow chamber assay as described in Procedure 1, Part2, Steps 16-21 (Figure 6).

Procedure 4: Myofibrils, Cardiac Muscle Tissue Homogenates & Synthetic Thick Filaments

Part 1: Sample Preparation

1 Prepare samples for use in chase analysis (Part 2). Follow option A for cardiac myofibrils, option B for cardiac muscle tissue homogenates, and option C to prepare full length myosin (FLM) extracts and use them to prepare HMM, S1 and synthetic thick filaments.

(a) Preparing Cardiac Myofibrils:

Timing 2 days

I. Isolate left ventricular tissue (Procedure 1, Part 1, steps 1-8) from hearts of desired species, suspend it in the Myofibril Relaxing Buffer with 1% Triton X-100 and subject it to homogenization using the OmniMixer homogeniser to remove the membranous layer.

II. Pass the crude homogenate through a 1000 µm polypropylene mesh sheet to remove larger particles and centrifuge the sample at 3500 rpm for 15 minutes.

III. Discard the supernatant, resuspend the pellet in Myofibril Relaxing Buffer with 1% Triton X-100, and repeat the homogenization step several times to obtain a clarified homogenate (usually 3-5 cycles).

IV. After the last homogenization step, resuspend the pellet in the Myofibril Relaxing Buffer and subject it to filtration using a 300 µm mesh filter followed by a 100 µm mesh filter to remove larger fragments.

V. Next, add the filtrate to a micro-fluidizer with the pressure set between 25000-35000 psi to break down and homogenize the particles on a micro-scale. Then, centrifuge the resulting sample at 3500 rpm for 15 minutes, collect a small sample of the supernatant and assess the concentration of myofibrils using a standard protein estimation assay (e.g. Bradford Assay). Using this measurement, resuspend the pellet this time in the experimental buffer of choice (SRX Experimental Buffer for either stopped-flow or plate-based assay; see Step 2) with 10% Sucrose. Note that the resuspension volume depends on the target myofibril concentration for downstream assays, ideally 10–12 mg/mL. Adjust the buffer volume accordingly and verify the final concentration for labelling and accurate loading in future experiments.

II PAUSE POINT Snap-freeze the myofibril sample in liquid nitrogen and store at -80˚C.

(b) Preparing Cardiac Muscle Tissue Homogenates

Timing 1 day

I. Weigh a known mass of left ventricular muscle tissue (Procedure 1, Part 1, steps 1-8) and suspend it in 15 volumes of Muscle Homogenization Buffer (e.g. 1 g of tissue in 15 mL) in an appropriately sized glass beaker.

II. Perform homogenization using Omni-Mixer at intermediate speeds (3000-4000 rpm) with alternate cycles of 10s run time and 20s of down time until the desired consistency is achieved (takes 2-3 cycles).

III. Centrifuge the muscle samples at 4000 rpm for 5-10 minutes at 4°C using the table-top centrifuge, discard the supernatant, and resuspend the pellet in 10 volumes of Muscle Homogenization Buffer and mix well. Repeat this cycle two more times to clear blood from samples.

IV. After the final centrifugation step, remove the supernatant and resuspend the pellet in 10 volumes of Muscle Homogenization Buffer, add 1% of Triton-X100, homogenize the sample using a dounce glass homogenizer. This step makes it quicker to dissolve the cellular membrane, providing a way to control the internal environment using a buffer of choice.

V. After glass homogenization, repeat the step 2-3 more times to remove Triton-X100 from samples. After the third centrifugation step, resuspend the pellet in 5 mL of the experimental buffer of your choice (SRX Experimental Buffer for either stopped-flow or plate-based assay;

see Step 2) for every 1 g of starting material to maintain compatibility of the buffer when conducting experiments.

VI. The resulting sample contains a mixture of larger and smaller size particles and to ensure consistent particulate size suitable for use in the assay, subject the sample to filtration using 100 µm mesh filter.

VII. Load equal amounts of samples and run an SDS-gel to determine the amount of myosin in each sample and perform a densitometric analysis of the myosin bands. Based on this quantification, calculate the appropriate loading amounts of each sample to normalize the amounts loaded between samples in the experiment.

(c) Preparing Full Length Myosin (FLM) from Muscle Tissue

I. ***Isolating FLM (Timing 3 days):*** On day 1, weigh a desired amount of cardiac tissue, dissect into smaller chunks, remove all visible fat, and pass the tissue through the meat grinder once.

II. Weigh the ground tissue and transfer it into a Myosin Extraction Buffer (3 ml/g) and mix for 20 minutes at 4˚C.

III. Spin using JS-4.2 centrifuge at 4000 rpm for 20 minutes at 4˚C, pass the supernatant through a Mira cloth and measure the volume.

IV. Precipitate myosin by adding 9 volumes of 10 mM $MgCl_2$ for every one volume of sample. Let the precipitate form and settle overnight to reduce the volume for centrifugation.

V. On day 2, siphon off as much of the volume above the precipitate as possible, spin the rest at 4000 rpm on a JS-4.2 centrifuge for 30 minutes at 4˚C and discard the supernatant.

VI. Weight the pellets, resuspend (1 g/ml) them in the High Salt Buffer and pass it through a dounce glass homogenizer.

VII. Measure the volume of the resuspended pellet and dilute the KCl to 0.3 M by using 3 volumes of ice-cold water, stir for 30 minutes to allow for equilibration and polymerization of actomyosin contaminant.

VIII. Spin using 250 ml bottles on Sorvall GSA centrifuge at 12000 rpm for 45 minutes at 4˚C to remove the contaminant actomyosin pellet.

IX. Measure the volume of the supernatant, add 5 volumes of BED buffer, and gently stir overnight at 4˚C to allow myosin to precipitate.

X. On day 3, spin using JS-4.2 centrifuge at 4000 rpm for 60 minutes at 4˚C. Discard the supernatant, weigh the pellet and resuspend it in equal volume (1 g/ml) of 2X Myosin Storage Buffer. Measure the myosin concentration and bring to 10-20 mg/ml using 1X Myosin Storage Buffer. This FLM preparation also serves as the starting material for generating other myosin forms—HMM and S1, as well as synthetic thick filaments—which can be used as alternatives in this assay.

- Proceed to Step XI to prepare HMM and S1 from FLM.

- Proceed to Step XVIII to reconstitute thick filaments from FLM.

⚠ **CRITICAL** Reconstituted thick filaments can be made from FLM by utilizing a specialized feature of its elongated tail region (LMM) (*2*, *3*). The LMM regions of myosins make electrostatic interactions with one another which aid them to align and aggregate as thick filaments. However, such electrostatic interactions among the tails are only productive in the storage buffer containing low ionic strength (low K+ and Cl- ions; < 150 mM KCl). To minimize such aggregation and to keep myosin fully soluble in solution, FLM is usually stored in a buffer of high ionic strength (high K+ and Cl- ions; > 300 mM KCl), which can be used to re-assemble thick filaments.

⏸ **PAUSE POINT** Snap-freeze in liquid nitrogen and store at -80˚C. Samples can be stored for 6–12 months with minimal loss of function.

XI. ***Preparing HMM and S1 from FLM (Timing 1 day):*** Thaw full-length myosin (FLM) in a 37 ℃ water bath until just melted. Precipitate the thawed FLM by adding 9 volumes of cold PM12 Buffer and allow to stand for 30 minutes at 4 ℃.

XII. Collect the precipitate by centrifuging at 5000 rpm for 30 minutes at 4 ℃. Discard the supernatant. Resuspend the pellet using a dounce homogenizer to ~20 mg/mL myosin concentration in the appropriate digestion buffer:

- **For HMM:** PIPES/KCl Buffer.
- **For S1:** PIPES/EDTA Buffer.

XIII. Starting with a 10 mg/ml fresh stock, prepare a 0.2 mg/mL solution of α-chymotrypsin or trypsin in the same buffer selected in Step XII. Add the protease solution to the myosin mixture to achieve a final protease concentration of 0.05 mg/mL. Incubate at room temperature with gentle swirling for the appropriate time:

- **For HMM:** 30 minutes
- **For S1:** 10-15 minutes

⚠ **CRITICAL** Trypsin or α-chymotrypsin acts to digest the myosin into its component peptides. The optimal trypsin concentration (typically 0.05 mg/mL) depends on the myosin source (skeletal or cardiac) and the desired fragment type (HMM or S1). A good practice is to do a pilot test with varying trypsin concentrations and incubation times to determine the optimal conditions for HMM or S1. The reaction progress should be carefully monitored, as overdigestion can produce smaller unwanted fragments. It is recommended to sample aliquots at different time points and analyze using SDS-PAGE to confirm the extent of cleavage and verify the presence of predominantly HMM (~350 kDa) or S1 (~110 kDa), as appropriate.

⚠ **CRITICAL** During step XIII, it is important to maintain a constant pH and ionic strength of the solution.

XIV. After digestion, quench the reaction by adding 1 mM PMSF stock solution (in DMSO) to achieve the target concentrations listed below, swirling gently to ensure even distribution. Incubate on ice for 30 minutes to ensure complete protease inactivation.
- **For HMM:** 0.3 mM PMSF
- **For S1:** 0.1 mM PMSF

XV. *(HMM only)* To separate HMM from LMM and undigested myosin, perform an additional overnight dialysis step with one buffer change before leaving it overnight. Use the **Low Salt Buffer** to reduce the final salt concentration to 20 mM KCl, promoting effective separation of LMM from HMM.

XVI. Add PMSF to achieve an additional final concentration of 0.1 mM, mixing gently to ensure even distribution (*for S1 only*). Centrifuge the mixture at 12,000 rpm for 45 minutes at 4 °C to remove undigested myosin and debris, leaving the HMM or S1 in the supernatant.

XVII. Carefully collect the supernatant and slowly add solid ammonium sulfate on ice with gentle stirring to reach 65% saturation (~430 g/L). Stir for 20–30 minutes at 4 °C to allow full precipitation.

XVIII. Centrifuge at 12,000 rpm for 45 minutes at 4 °C to pellet the precipitated HMM or S1. Discard the supernatant. Resuspend the pellet in cold PM12 Buffer.

XIX. Dialyze the resuspended sample against 15 L of PM12 Buffer at 4 °C for 48 hours with at least two buffer changes per day.

XX. After dialysis, clarify by centrifuging at 35,000 rpm for 2 hours at 4 °C. Add solid sucrose slowly while stirring in the cold room to achieve 10% (w/v) final concentration.

XXI. Measure the concentration using nanodrop or Bradford assay. Snap-freeze using liquid nitrogen and store at -80˚C. Label each container with the measured concentration and production date.

⏸ PAUSE POINT Frozen reagents remain stable for 6–12 months with minimal loss of function. It is recommended to periodically assess HMM and S1 quality using functional assays (e.g., ATPase activity).

XXII. ***Preparing Reconstituted Thick Filaments (Timing 3 hrs):*** Thaw a desired amount of FLM stored in Myosin Storage Buffer.

XXIII. Keeping in mind the starting concentrations of myosin and KCl in the storage buffer, dilute the sample appropriately using the Myosin Storage Buffer (0.6 M KCl) and/or Thick Filament Buffer (0 M KCl) to attain the desired final concentrations of the agents. For example, if the start-up concentration of FLM in the storage buffer is 8 mg/mL (16 µM assuming 500 kDa as the mol wt.), dilute the myosin sample by 20-fold using Thick Filament Buffer alone to attain final concentrations of myosin and KCl as 0.8 µM and 30 mM, respectively. If the start-up myosin concentration is greater than 8 mg/ml, use Myosin Storage Buffer to first adjust the myosin concentration to 8 mg/ml and then use Thick Filament Buffer to further dilute this sample by 20-fold to attain myosin and KCl concentrations of 0.8 µM and 30 mM, respectively.

⚠ CRITICAL The concentrations detailed here are sensitive to instrument settings (such as instrument type, excitation/emission wavelengths) and to the proportion of active myosin in the sample. These factors should be carefully considered and optimized under your specific experimental conditions to ensure accurate and reliable results.

XXIV. Following dilution, incubate the myosin sample on ice for 2 hours to allow thick filament formation. Filaments prepared this way are intended for same-day use only and are not recommended for storage overnight.

Part 2: Chase Assay

2 To perform a stopped flow instrument based chase assay, follow option A. To perform a 96-well plate based chase assay, follow option B.

(a) Stopped Flow Instrument-based Assay

Timing 1hr per sample

⚠ **CRITICAL** These steps require familiarity with stopped-flow instrumentation and specialized expertise to perform these experiments successfully.

! TROUBLESHOOTING

It is important to note that muscle tissue homogenates and myofibrils are larger in size than other myosin models (STF, HMM, S1) and can clog the narrow channels of stopped-flow instruments, making them unsuitable for single-turnover kinetics. This limitation can be circumvented by using a plate-based assay, discussed below (option b).

i. This assay is performed on a double-mix stopped flow instrument at room temperature that employs three syringes. Prepare 3 syringes containing the following components in the SRX Experimental Buffer.
   - Syringe-1: Buffer containing 400 nM myosin.
   - Syringe-2: Buffer containing 400 nM Mant-ATP
   - Syringe-3: Buffer containing 4 mM ATP

⚠ **CRITICAL** If you intend to study the effect of ionic strength on the myosin SRX state, you may adjust the SRX Experimental Buffer to reflect varying amounts of KCl, as desired.

⚠ **CRITICAL** When working with therapeutic agents, a 1-2% DMSO (± agent) is recommended in all syringes.

⚠ **CRITICAL** Myosin S1 and HMM can also be tested using the same procedure but care should be taken to account for their molecular weights to attain a myosin concentration of 400 nM in the Syringe-1.

⚠ **CRITICAL** When working with myofibrils, assume that 40% weight of myofibrils comes from myosin alone and accordingly load an appropriate amount of the stock in the Syringe-1 in the place of purified myosin reagent. A rough guide of approximate molecular weights and myosin content estimates for different models is detailed as follows: *S1*, ~110 kDa; *HMM*, ~350 kDa; *Full-Length Myosin (FLM)*, ~500 kDa; *Myofibrils*, Complex assembly – assume ~40% of total mass is myosin (e.g., 1 mg myofibrils ≈ 0.4 mg myosin); *Cardiac Muscle Tissue Homogenate*, Mixed cellular components – assume ~10–15% of total mass is myosin (e.g., 1 mg homogenate ≈ 0.1–0.15 mg myosin). For greater accuracy, run an SDS-PAGE of the sample alongside known myosin standards at various dilutions to estimate myosin content precisely.

ii. Mix equal volumes (10 µL each) of samples in syringes 1 and 2 and incubate for 20s to allow Mant-ATP binding and hydrolysis of Mant-ATP to inorganic phosphate (Pi) and Mant-ADP.

iii. Chase by adding 20 µl of the buffer from syringe 3 containing non-fluorescent (dark) ATP to the mixture in step ii. The concentrations of myosin, Mant-ATP, and non-fluorescent ATP attained in the final mixture are 100 nM, 100 nM, and 2 mM, respectively

iv. Monitor the resulting fluorescence decay due to Mant-nucleotide dissociation from myosin over a course of time varying from 10 to 30 minutes. . The excitation and emission wavelengths of Mant-nucleotide are 365 nm and 450 nm, respectively. The sampling rate should be adjusted to 0.25-0.5kHz for the first 20s to capture the initial faster decline in the fluorescence signal and it can be then lowered to 0.1kHz for the rest of the duration to acquire the slower phase.

⚠ **CRITICAL** It is important to emphasize here that the total acquisition time should be set sufficiently long to capture most of the slower phase for a more accurate determination of myosin SRX population.

(b) 96-well plate-based assay

Timing 1-2hrs

⚠ **CRITICAL** Akin to the stopped-flow assay, single turnover experiments can also be performed in a 96-well plate. It is important to note that all the previously described experimental models (S1, HMM, STFs) as along with myofibrils and muscle tissue homogenates can be tested using the plate-based method, expanding its utility across diverse sample types. The same format as in the stopped-flow assay (option a) can be used with all of these models, as detailed below.

⚠ **CRITICAL** When muscle tissue homogenates are to be used, matching myosin concentration between tissue samples becomes important. Therefore, the best practice is to run the SDS-gel by loading equal amounts of prepared samples along with a myosin of known concentration to roughly estimate the myosin concentration in each sample. This can be used as a guide to appropriately load each sample to normalize the myosin concentrations between samples.

i. Prepare the set amount (e.g. 10 mL) of sample by diluting myosin to a concentration of 100 nM in the SRX Experimental Buffer and load 100 µL of this sample to each well of a 96-well plate.

ii. Add appropriate amount of Mant-ATP stock (usually 5 mM) to attain 100 nM in each well and incubate the mixture for 20s.

iii. After 20s, add appropriate amount of non-fluorescent (dark) ATP stock (usually 100 mM, pH 7) to attain a final concentration of 2 mM in each well, gently mix the sample, and run a kinetic scan at a frequency of 5-10 Hz for a minimum of 60 minutes or until enough data points have been collected so that a reproducible distribution of myosin DRX and SRX state(s) can be measured. The excitation wavelength could be anywhere from 365 nm to 405 nm while the emission is usually monitored at wavelengths between 450 and 475 nm.

! TROUBLESHOOTING

iv. Conduct separate experiments by using the same combination of myosin and Mant-ATP concentrations in step-3 with no dark ATP addition and measure the fluorescence signal at time = 0 s and at time = ∞. Normalize the kinetic traces acquired in the presence of dark ATP with those obtained in the absence of dark ATP.

v. Fit the normalized fluorescence decay traces to a biexponential decay function to yield the amplitudes and rates of fast (DRX) and slow (SRX) phases. See Data Analysis Section (Procedure 5) for more details.

vi. If one wants to directly report DRX and SRX proportions, then the fraction of non-specifically bound Mant-ATP in a sample/tissue type must be measured. To do this one performs a series of chase experiments that are fit to a single exponential decay (Figure 11), see Box1 of additional procedures for a detailed protocol.

Box 1: **Additional Procedures**

Calculating the proportion of fluorescence decay attributable to non-specifically bound Mant-ATP washout

To quantify the fraction of fluorescence decay that can be attributed to the washout of non-specifically bound Mant-ATP, researchers should perform a competition assay (Figure 11).

1. Prepare samples as previously described across procedures 1-4.
2. Incubate samples in rigor buffer solution containing 250uM Mant-ATP and increasing concentrations of ATP (0 mM-4 mM) for 15 minutes, acquiring a fluorescent image every 30 seconds. Run all concentrations of ATP on the same slide, starting with the lowest and building up.
3. Measure the fluorescence intensity of the tissue sample and background using an ROI in FIJI as described in the data analysis supplement.
4. Subtract background from the tissue intensity for each frame in each video.
5. Average the last three frames to obtain the mean plateau fluorescence intensity
6. Normalise the intensity of each ATP concentration plateau to the baseline intensity of the image after incubation with only Mant-ATP (0 mM ATP) between 1 and 0.
7. Fit the normalised fluorescence to an unconstrained single exponential decay to obtain the proportion of fluorescence decay attributable to the washout of non-specifically bound Mant-ATP, as the plateau of the curve (i.e. ymin).

Photobleaching

It is also necessary to quantify the fraction of fluorescence decay that can be attributed to photobleaching. Previous authors have shown this to be a negligible fraction, but researchers should verify this with the specific equipment and configuration used in their experiment. To test for photobleaching, researchers can incubate a sample with Mant-ATP solution as normal and record fluorescence decay over a 20-minute period (10 ms/5 s).

[Production: end of Box 1]

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2: Troubleshooting table.

Step	Problem	Possible Reason	Possible Solution
Procedure 1: part 2: Cardiac Tissue/iPSC-CM Flow Chamber Buffer Infusion Step 20.	Sample instability during chamber flushes	The size of the two small adhesive dots will heavily influence the volume of the chamber	We recommend aiming for the smallest possible dots of adhesive which stably secure the pins in

		and the stability of the sample under the pins.	place to reduce the chamber volume and to allow the cover slip to press down on the sample.
Procedure 3: part 1:iPSC-CM Differentiations; Step 16	Excess cell death or differentiation failure	CHIR99021 concentrations will require optimisation for different cell lines to get the right balance of death vs differentiation.	10 µM is a recommended starting point for most human iPSC lines. 4-20 µM can be trialled.
Procedure 4: part 2: Cardiac Muscle Homogenate SRX:DRX Assay; Step 2	Muscle tissue homogenates may clog the narrow channels of the stopped-flow instrument, making them not amenable for the single turnover kinetics on a stopped-flow instrument.	Muscle tissue homogenates are too large for the stopped-flow instrument	This issue can be circumvented by using a plate-based assay
Procedure 3: part 2: Fluorescence Acquisition (Cardiac tissue, single fibres, iPSC-CMs). Step 34.	Sample shrinking or failure to obtain a two-phase decay	Laser intensity, exposure time and interval time may require optimisation for each microscope setup	Adapt frequency of acquisition and length of exposure. We recommend 5 second intervals with 20ms acquisition.
			.

[H1] Timing

[H2] Cardiac Tissue:

Procedure 1: Steps 1-8: Tissue Dissection: 30 minutes

Procedure 1: Steps 9-11: Tissue Skinning: 7 hours

Procedure 1: Steps 12-15: Tissue Dissection & Slide Preparation: 40 minutes

Procedure 1: Steps 16-21: Fluorescence Acquisition: 30 minutes per slide

[H2] Single Fibres:

Procedure 2: Steps 1-7: Tissue Dissection & Fibre Preparation: 3 days

Procedure 2: Steps 8-13: Fluorescence Acquisition: 30 minutes per slide

[H2] iPSC-CMs:

Procedure 3: Steps 1-26: Differentiations: 1-2 months

Procedure 3: Steps 27-33: Tissue Dissection, skinning and slide preparation: 40 minutes

Procedure 3: Step 34: Fluorescence Acquisition: 30 minutes per slide

[H2] Myofibrils:

Procedure 4a: Step 1-6: Preparing myofibrils: 2 days

[H2] Cardiac Muscle Tissue Homogenates

Procedure 4b: Steps 1-7: Prepare cardiac muscle tissue homogenates: 1 day

[H2] Synthetic Thick Filaments:

Procedure 4c: Steps 1-10: Isolating Full Length Myosin: 3 days

Procedure 4c: Steps 11-17: Preparing HMM & S1: 1 day

Procedure 4c: Steps 18-20: Preparing reconstituted thick filaments: 3 hours

[H2] Fluorescence Acquisition:

Procedure 4 Part 2a: Steps 1-4: Stopped Flow Instrument Fluorescence Acquisition: 1 hour per sample

Procedure 4 Part 2b: Steps 1-5: 96-well plate-based Fluorescence Acquisition: 1-2hrs

[H1] Anticipated Results

The Mant-ATP assay can provide an assessment of the relative abundance of myosins in the SRX and DRX states in permeabilized tissue, cells and isolated proteins. For example, Toepfer et.al. used the assay with mouse left ventricular myocardial tissue to investigate the effect of HCM-linked *MYH6* missense variants on SRX proportions[16]. They compared the proportions of myosins in the SRX state(s) in *MYH6* variant mice to wildtype mice. Mant-ATP assays were performed on 3 independent heart tissue samples, from which three regions of each sample were sampled for fluorescence decay. Fluorescence decay data was then fitted to a double-exponential decay function to obtain values of P2. After correction for the removal of non-specific fluorescence decay, SRX proportions were obtained for each ROI, from which means were calculated for each genotype.

Wildtype mice samples exhibited a mean SRX proportion of 66.6%, whilst $MYH6^{R403Q/+}$ mice exhibited a lower mean SRX of ~60.3%. The SRX proportion of wildtype mice samples had a standard deviation of 3.4, whilst $MYH6^{R403Q/+}$ variant mice samples had a standard deviation of 2.7. This experimental setup was sufficiently powerful to detect a decrease in SRX proportions in the mutant mice of

approximately 6%, with a significance level less than 0.03. Significance was assessed using a 1-way ANOVA and post hoc Bonferroni test. The difference was reversed following treatment with mavacamten where Mavacamten treated cells had an SRX of 74% with a standard deviation of 2.9[16]. This variability is broadly consistent across assay settings, but some increased variability can be observed depending on the person performing the experiments and their experience in running the assay. For this reason it may be necessary to increase the power of the experiment by increasing the number of samples used. Across the literature in general the assay is sufficiently powered with three biological replicates and 4-6 technical replicates from each biological replicate.

Assessing biological and technical variance across species shows that there is a consistent variability across two separate data sets that contain 5 biological replicates with 4 technical replicates in each biological replicate from both mouse and pig left ventricular samples (Supplemental Excel file, and Figure 12). Where mouse samples show a standard deviation of 6% across a 20-measurement data set, and pig samples show a standard deviation of 5.9%. Together this data show that mouse and pig left ventricular tissues have statistically different SRX proportions where mouse show on average a mean and standard error of 74.5% ± 1.3% of SRX myosin, which in pig tissue is 67.8% ± 1.3%. Using a student's T-test this shows that these samples are deemed to be significantly different with p = 0.002 (Figure 12B).

In combination this data and the published data-sets highlight the power of using the Mant-ATP assay in both disease modelling and drug evaluation. Importantly, we caution against interpreting the SRX and DRX values obtained as the true proportions of SRX and DRX in living tissue but instead highlight the utility of the Mant-ATP assay for comparisons to illustrate how SRX:DRX proportions can vary in disease or under drug treatment within or across model systems.

Data Availability

All data used to produce this protocol are available as supplemental files or by request to the authors.

Author Contributions: S.T.M.J and C.N.T oversaw compilation of the manuscript. S.T.M.J, F.E.PC, Y.P and V.S compiled the protocol for iPSC-CMs. S.T.M.J, F.E.PC, P.R and M.S compiled the protocol for cardiac tissue. E.G.M and J.O compiled the protocol for single fibres. S.K.G and S.N compiled the protocols for myofibrils, fibre bundles, muscle tissue homogenates and isolated myosin. All authors contributed to editing the manuscript.

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Competing Interests:

C.N.T. is a member of the Scientific Advisory Board of Cardiatec, who were not involved in this study. The authors declare no other competing interests.

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Figure legends:

Figure 1: Illustration of the Mant-ATP experiment and applicable assay types. a) Diagram of the workflow of the Mant-ATP assay. Myosin is first washed of residual nucleotides and then saturated with Mant-ATP (blue), which increases in fluorescence upon specific binding with myosin. Mant-ATP is then chased out through a subsequent incubation with “dark” ATP (black). **b)** Diagram depicting the range of model systems that the Mant-ATP assay can be used with to determine SRX:DRX proportions, ranging from permeabilised muscle tissue to purified myosin fragments.

Figure 2. A Normalized Mant-ATP decay curve after addition of dark ATP: Normalised fluorescence is fitted to a two phase decay function where $Normalised\ Fluorescence = 1 - P_1\left(1 - \exp^{\left(-t/T_1\right)}\right) - P_2\left(1 - \exp^{\left(-t/T_2\right)}\right)$. P1 corresponds to t1he proportion of fluorescence decay attributed to the fast phase, with decay constant (T1), comprised of fluorescence decay from Mant-ATP released from DRX myosins, and non-specifically bound Mant-ATP. P2 corresponds to the proportion of fluorescence decay occuring during the slow phase. P2 is adjusted for the fluorescence decay of non-specifically bound Mant-ATP, obtaining the proportion of SRX myosins. DRX = 1 – SRX.

Figure 3: Building the Mant-ATP flow chamber. a) Schematic diagram depicting the layout of the flow chamber used for permeabilised muscle tissue samples. **B)** layout of the flow chamber used for permeabilised iPSC-CMs.

Figure 4. Preparing Single Fibre Slides. Isolated fibers are mounted on halved transmission electron microscopy copper grids (200 mesh x 125 µm pitch) glued on regular microscope slides.

Figure 5: Flow chamber tissue mounting. Tissue is securely mounted under the two pins within the flow chamber.

Figure 6: Experimental loading order of the Mant-ATP flow chamber. Schematic depicting the chamber infusions used for the Mant-ATP assay with permeabilised muscle tissue samples. After permeabilization, the chamber is infused with glycerinating solution for storage. The chamber is then infused with rigor buffer for 2 sets of 5 minutes. The chamber is then infused with Mant-ATP solution for 10 minutes, during which fluorescence uptake is recorded. The chamber is then infused with ATP chase solution for 10 minutes, during which fluorescence decay is recorded.

Figure 7: Dissecting Single Fibres: Biopsis are cut longitudinal to the direction of the fibers, then skinned and dissected to single fibers. The pull the fibers from a bundle the tip of a needle can gently hold the biopsy in place (do not poke holes or compress the fibers). Using a second needle use the tip to poke right at the edgesof the fiber and pull it gently away from the bundle. You can poke through the ends as they will be pressed by the grid hinges later.

Figure 8: Assembling the Mant-ATP flow chamber for fiber experiments. Add a small droplet of relaxing solution on top of the grid and transfer the isolated fibers. Use the tip of a 25G needle to raise the square ends of the grid from the bottom; they can now be used to pin down the fibers at each end, by carefully pushing them on top of the fiber. After 6 fibers have been mounted, a chamber is created around the grid with sticky tape and a coverslip. Press lightly on the coverslip at the position of the tape to avoid breaking.

Figure 9: Imaging the fiber flow chamber. The imaging zone is limited to half of the grid at a time, as this fits within the field of view of a 10x lens. Note which half was imaged first. No difference has been found between the two sides after imaging.

Figure 10: Overview of the preparation of Mant-ATP flow chambers using iPSC-CMs. a) Schematic diagram depicting the steps involved in harvesting iPSC-CMs and mounting them in the flow chamber. **b)** Photos demonstrating the process of mounting the iPSC-CM sample under the pins within the flow chamber. **C)** Photos showing iPSC-CMs securely mounted under the three pins within the flow chamber.

Figure 11: Establishing the Mant-ATP correction factor for calculating SRX:DRX. Normalised fluorescence from competition reactions between Mant-ATP and increasing concentrations of ATP for specific binding sites on myosin heads. Data are fitted to a single exponential decay curve, with the plateau indicating the percentage of fluorescence that can be attributed to non-specifically bound Mant-ATP, which should be used to adjust P2 to obtain fluorescence decay attributable to SRX myosin. (N=5, errors bars correspond to 95% confidence interval).

Figure 12: Assessing variability across biological and technical replicates A) Data showing individual biological and technical replicates of SRX proportions across mouse and pig left ventricular tissue. B) Collated data showing mouse and pig data from two separate datasets plot together and compared

by students t-test indicating that mouse and pig left ventricular tissue have significantly different SRX proportions. N = 20 across 5 biological replicates for mouse samples and N = 20 across 5 biological replicates for pig samples.