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Measuring passive myocardial stiffness in *Drosophila melanogaster* to investigate diastolic dysfunction

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Abstract

Aging is marked by a decline in LV diastolic function, which encompasses abnormalities in diastolic relaxation, chamber filling and/or passive myocardial stiffness. Genetic tractability and short life span make *Drosophila melanogaster* an ideal organism to study the effects of aging on heart function, including senescent-associated changes in gene expression and in passive myocardial stiffness. However, use of the *Drosophila* heart tube to probe deterioration of diastolic performance is subject to at least two challenges: the extent of genetic homology to mammals and the ability to resolve mechanical properties of the bilayered fly heart, which consists of a ventral muscle layer that covers the contractile cardiomyocytes. Here, we argue for widespread use of *Drosophila* as a novel myocardial aging model by (1) describing diastolic dysfunction in flies, (2) discussing how critical pathways involved in dysfunction are conserved across species and (3) demonstrating the advantage of an atomic force microscopy-based analysis method to measure stiffness of the multilayered *Drosophila* heart tube *versus* isolated myocytes from other model systems. By using powerful *Drosophila* genetic tools, we aim to efficiently alter changes observed in factors that contribute to diastolic dysfunction to understand how one might improve diastolic performance at advanced ages in humans.

Keywords: *Drosophila* • heart • aging • myocardial stiffness • diastolic dysfunction • atomic force microscopy

Introduction: determinants of diastolic dysfunction

Cardiac output is determined by the volume of blood ejected with each heartbeat and by the heart rate. While ventricular contractile force helps to determine ejection fraction during systole, proper myocardial relaxation during diastole is required for appropriate filling. Both pumping and filling properties of the heart can affect cardiac output, overall cardiac performance and can independently lead to heart failure when perturbed [1].

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The symptoms of heart failure observed in nearly half of all patients are associated with impaired ventricular relaxation or diastolic dysfunction [1–3]. Relaxation and filling abnormalities associated with diastolic dysfunction often result in elevated LV end-diastolic pressures and can compromise overall work output of the heart [4–6]. Various pathogenic mechanisms are believed to underlie diastolic dysfunction. In addition to impaired relaxation, these include perturbations that decrease myocardial distensibility and/or that increase LV end-diastolic stiffness. The molecular basis of these altered diastolic indices encompasses aberrant Ca^{2+} handling, extracellular matrix (ECM) modifications and myofilament dysfunction [3–5, 7]. In failing hearts, perturbed Ca^{2+} homeostasis can contribute to diastolic dysfunction (Fig. 1). Slowed Ca^{2+} transients can attenuate myocardial relaxation rates, prolong active force generation and hinder ventricular filling. Changes in Ca^{2+} handling result from altered expression of Na/Ca^{2+} exchangers, the sarcoplasmic reticulum Ca^{2+} uptake protein SERCA, phospholamban (a SERCA regulator) and ryanodine receptors [3–5, 7]. Furthermore, post-translational modification of these proteins by numerous protein kinases can influence their activity. Elevated diastolic Ca^{2+} levels can also influence overall ventricular distensibility by potentially increasing the active tone of resting myocytes [4].

Modifications in cytoarchitectural components are likewise considered critical determinants of diastolic performance and failure (Fig. 1). For example, the mechanical attributes of the ECM that surrounds individual myocytes impact the overall compliance of the myocardium [8]. Extracellular matrix material properties are largely influenced by the absolute quantity and distribution of collagen, the ratio of different collagen types and isoforms and the extent of post-translational modification [3–5, 7]. Furthermore, deposition of advanced glycation end products can augment collagen cross-linking, alter the physical properties of the ECM and increase LV diastolic stiffness [4, 5, 7].

Dysfunction in the myofilamentous components of the cytoarchitecture can additionally initiate abnormal cardiomyocyte compliance and relaxation (Fig. 1). Passive properties of titin are believed to impart the majority of the passive tension characteristics of the ventricles. Hence, alterations in the connecting (titin) filaments and in proteins of the thick and thin filament complexes can result in impaired diastolic function [3, 4].

Thus, a multitude of mechanisms appear to be associated with the development of diastolic dysfunction. Many aspects of diastole can be altered either alone or in combination to elicit irregular performance [4]. Comprehending the factors that predispose one to diastolic dysfunction and developing new genetic models that permit detailed quantitative analysis and descriptions of its biochemical and biophysical characteristics should provide important insights into diastolic heart failure and potentially facilitate the development of targeted treatments.

Diastolic dysfunction: effect of age and models for investigation

Diastolic dysfunction is a major cardiac deficit that can result in diastolic heart failure. There appear to be several predisposing factors for diastolic dysfunction including female gender, obesity, coronary artery disease, hypertension, diabetes mellitus and importantly, age [4]. Senescent hearts exhibit both impaired relaxation and increased myocardial stiffness [7]. Aging is associated with increased interstitial fibrosis and collagen cross-linking, disturbed Ca^{2+} homeostasis and altered expression and modification of myofilamentous components, which, as outlined above, are major contributors to impaired diastolic function. However, the specific causes of age-dependent changes in diastolic performance remain difficult to study. This can be attributed to a paucity of animal models that recapitulate human diastolic dysfunction and particularly myocardial stiffening, which is often unaffected in murine models despite extensive matrix, myofibrillar or signalling abnormalities [4, 5]. Furthermore, the life span of vertebrate models is often several years, making senescent-related studies prohibitively lengthy. Conversely, *Drosophila melanogaster*, the fruit fly, is a relatively inexpensive, rapidly aging, genetically tractable organism that is gaining acceptance as a viable alternative for investigating heart development, cardiac pathophysiology and cardiac senescence [9–13].

Evidence of age-associated diastolic dysfunction in *Drosophila*

The advent of new dissection techniques, imaging assays and analysis protocols has provided a much broader understanding of and appreciation for the utility of the simple bilayered *Drosophila* heart tube (Fig. 2A–C) as a model for studying apparently conserved responses to mutations in cardiac components and to age [11, 12]. Using a semi-automated heart analysis program, Cammarato *et al.* [14] showed that wild-type *Drosophila* hearts exhibit a steady decrease in mean diastolic and systolic diameters across the tubes over time (Fig. 2D). Furthermore, progressive decline in diastolic dimensions was significantly greater than that for systolic dimensions. This highlights deterioration in contractile performance with age as indicated by significantly reduced per cent fractional shortening. It is obvious that in senescent (5-week-old) flies, there is a substantial attenuation of diastolic performance relative to that of juvenile (1-week-old) flies. This senescent-dependent response is indicative of the impaired myocardial relaxation and possibly of cardiac chamber stiffening as normally observed during age-associated human diastolic dysfunction.

Conservation of components involved in diastolic dysfunction in flies

The responses to mutations and age observed in the *Drosophila* heart suggest that the fly may serve as a powerful tool for studying cardiac molecular control mechanisms and basic physiological processes that have been conserved during evolution [9–13]. These fundamental responses and processes depend upon conserved transcriptomic and proteomic networks. The extent of conservation with higher organisms has been described by Bier and

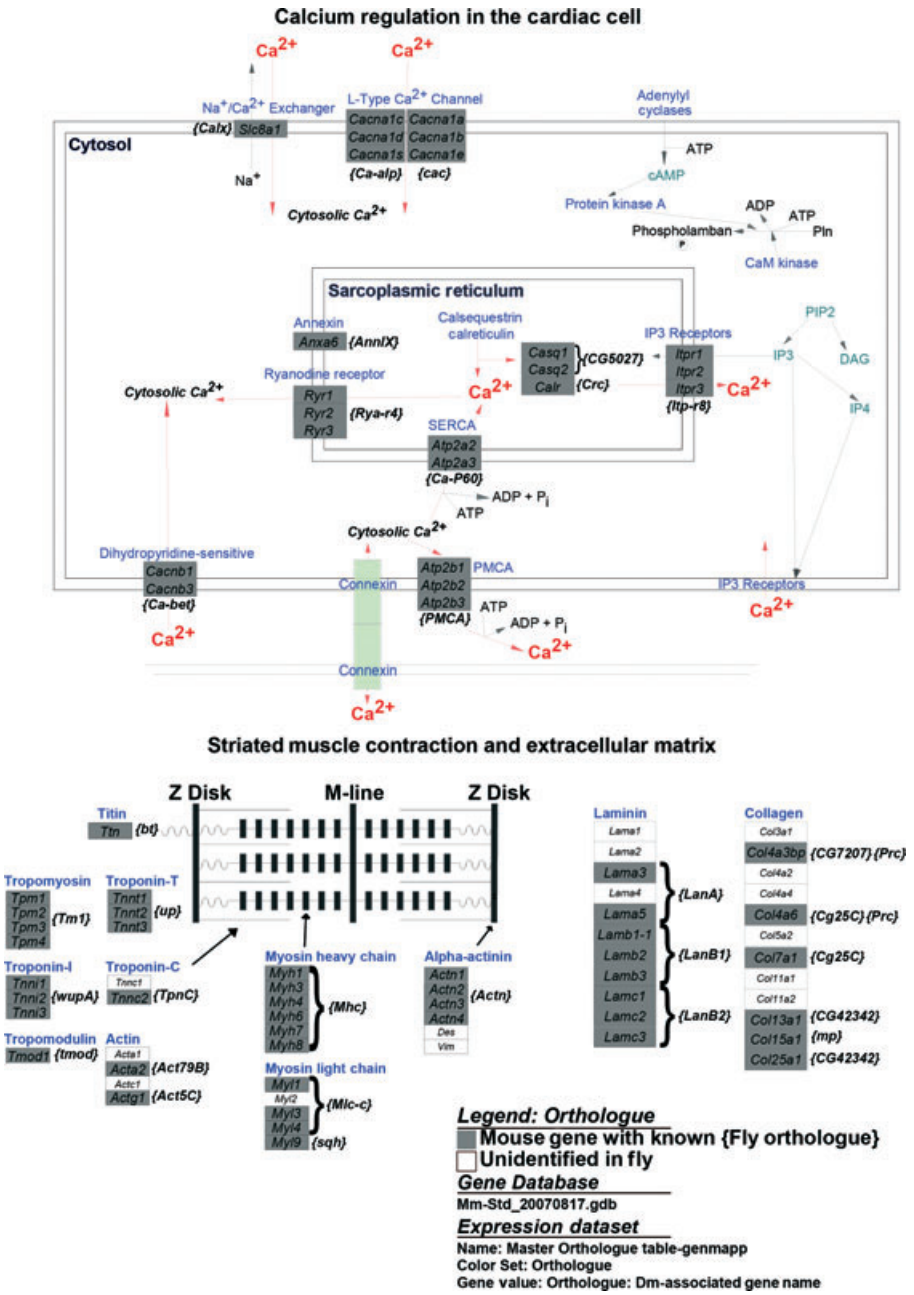
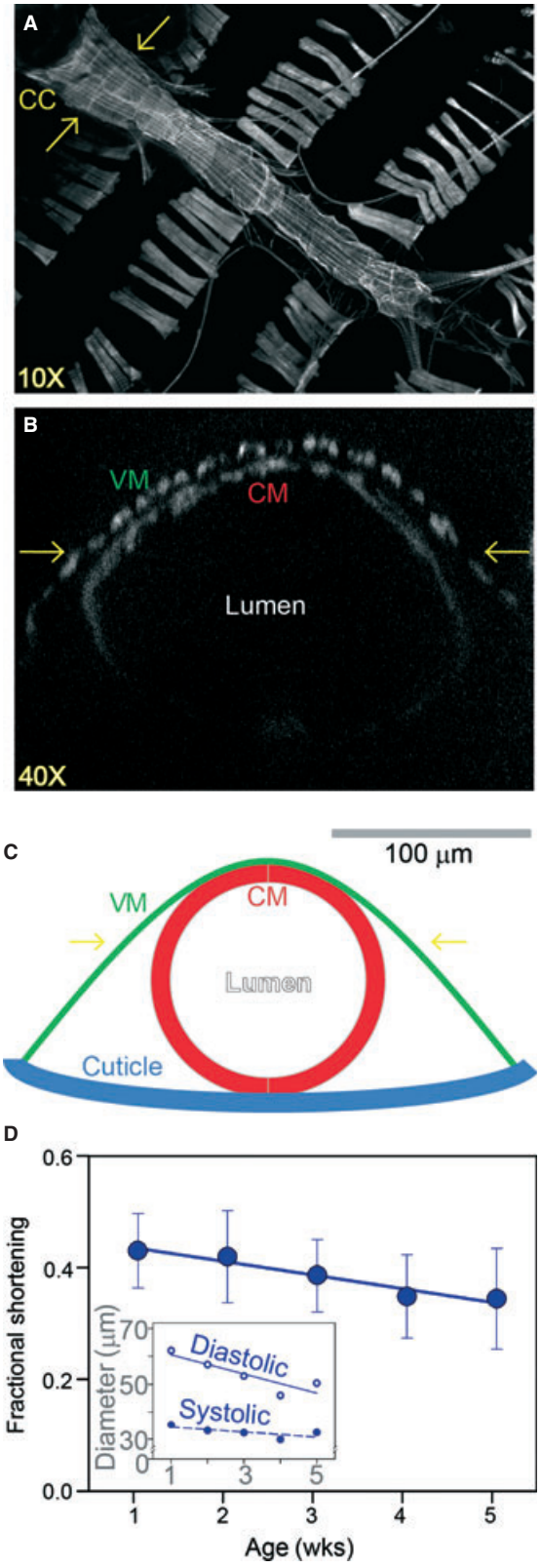


Fig. 1 Cardiomyocyte gene pathways associated with diastolic dysfunction. Top: In failing hearts, perturbed Ca^{2+} homoeostasis can contribute to diastolic dysfunction. Many genes involved with Ca^{2+} handling within cardiomyocytes are conserved between *Drosophila* and mammals. Here, we compiled a gene orthology database from NCBI homologene [16], Ensembl [17], and InParanoid (used by FlyBase to assign orthologues [18]). This database assigns *Drosophila* orthologues to 12,304 unique mouse genes and was used to visualize orthologues related to Ca^{2+} homoeostasis. Examples of orthologous genes are shown in grey boxes with the fly gene listed next to each box. Such conservation suggests that changes in expression of Ca^{2+} handling genes could equally affect diastolic performance of both flies and mammals. Bottom: Modifications in cytoarchitectural components are also critical determinants of diastolic performance and diastolic heart failure. Examples of orthologues related to striated muscle contraction and to the extracellular matrix are shown. Such conservation suggests that changes in expression of these cytoarchitectural genes could have profound consequences on diastolic performance of both flies and mammals. The presence of mRNA or proteins encoded by many of the *Drosophila* genes listed has been directly confirmed by cardiac specific microarray experiments (Anthony Cammarato, Alexander C. Zambon, Sanford I. Bernstein, Rolf Bodmer, unpublished data) and by proteomic assessment [15].



Bodmer, who suggested that nearly 75% of human disease genes, which include those that cause cardiovascular disorders, have homologues in *Drosophila*. Cammarato *et al.* [15] recently extended this finding to the proteome, which revealed a substantial degree of conservation between fly and murine hearts, especially with cytoarchitectural components.

As an example of pathway level conservation found between *Drosophila* and mammals, we compiled a gene orthology database from three independent publicly available sources: NCBI homologene [16], Ensembl [17] and InParanoid (used by FlyBase to assign orthologues [18]) (Fig. 1). This database assigns *Drosophila* orthologues to 12,304 unique mouse genes and was used to visualize orthologues of three signalling networks related to passive myocardial stiffness and diastolic performance: calcium regulation in the cardiac cell, the ECM and striated muscle contraction (Fig. 1). These pathways were downloaded from wikipathways [19] and coloured with the GenMAPP program [20]. Conservation and reduced redundancy in flies of most major pathway components in all three networks are clearly evident. Ongoing cardiac-specific microarray experiments have confirmed the expression of the majority of these orthologous genes (Anthony Cammarato, Alexander C. Zambon, Sanford I. Bernstein, Rolf Bodmer, unpublished data). Subsequent extension of this analysis will identify differentially expressed cardiac gene candidates with age and provide therapeutic targets that can be rapidly and systematically manipulated in flies to determine novel roles in diastolic relaxation and in cardiac stiffness, *in situ*.

Measuring passive myocardial stiffness *in situ* in flies

Mechanical analyses have given remarkable insight into the role myocardial stiffness plays during development [21, 22] as well as during aging and pathogenesis [23, 24]. In particular, atomic force microscopy (AFM)-based indentation approaches, in which the tissue of interest is indented and the reaction forces are measured, offer the ability to investigate myocardial micromechanical proper-

Fig. 2 The *Drosophila* heart and evidence for age-associated diastolic dysfunction. (**A**) The *Drosophila* heart lies along the dorsal midline of the abdomen. It consists of a simple linear tube composed of a single layer of contractile cardiomyocytes covered by a thin ventral longitudinal muscle layer. Fluorescent image modified from [15]. The conical chamber (CC) is the most pronounced muscular region of the heart and is likely a primary determinant of circulatory flow. (**B**) Cross-section through the CC revealing the location of the ventral muscle layer (VM) and underlying cardiomyocytes (CM). (**C**) Illustration depicting the layers of the myocardium shown in (**B**). (**D**) Fractional shortening of the *Drosophila* heart tube declines with age. This results from an accelerated decline of diastolic diameters over time relative to the decline in systolic diameters. Panel adapted from Ref. [14]. The rapid deterioration of diastolic diameters suggests senescent-associated diastolic dysfunction as found in higher organisms.

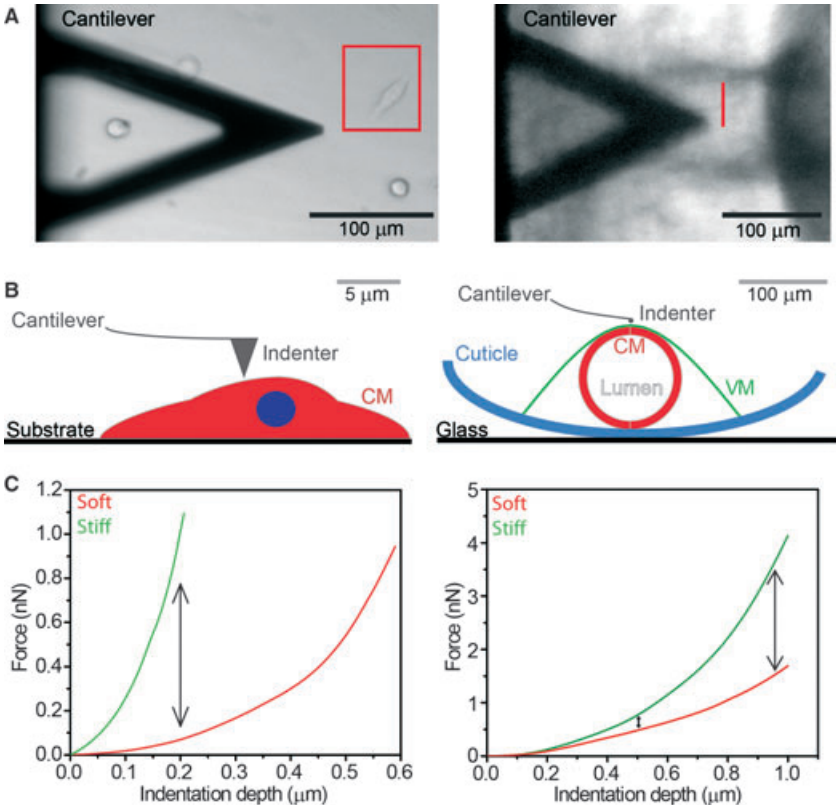


Fig. 3 Direct mechanical comparison of cultured murine myocytes and fly heart tubes. (**A**) Brightfield images of an AFM cantilever (open triangular-shaped object) positioned over a mouse myocyte (left) and the *Drosophila* heart tube (right). Note that the box on the left image highlights a probed region where the myocyte is attached and the line on the right image highlights the midline to lateral edge of the fly heart tube that is typically probed using our analysis method. (**B**) Schematics of a cross-section of the biological specimens analysed by AFM (mouse on left, fly heart tube on right). Note that each one is drawn to its own scale as indicated in the top right of each illustration. (**C**) Left: representative AFM force-indentation curves plotted for mouse cardiomyocytes cultured on a stiff glass coverslip (green) *versus* a soft hydrogel (red). Arrow indicates an increased reaction force at 200 nm indentation depth consistent with a more rigid remodelling response due to substrate stiffness. Right: force-indentation plots for two fly heart tubes (red *versus* green curve) indicating overall differences in myocardial stiffness. Left arrow depicts a difference in reaction force response at shallow indentation depth (<500 nm) while the right arrow reveals a difference in force response at deep indentation depth (>1 μm), reflecting disparity in ventral muscle layer and cardiomyocyte stiffness, respectively. All nanoindentation was performed with an MFP-3D Bio AFM (Asylum Research, Santa Barbara, CA, USA) mounted on a Ti-U fluorescent inverted microscope (Nikon Instruments, Melville, NY, USA) with Au-coated pyramid-shape tips (TR400PB; Olympus, Center Valley, PA, USA) for mouse cardiomyocytes and 2 μm radius borosilicate spheres (120 pN/nm; Novascan Technologies, Ames, IA, USA) for *Drosophila* heart tubes.

ties [25]. Atomic force microscopy-based analyses have revealed that cardiomyocytes stiffen with age [26], suggesting that senescent-associated diastolic dysfunction may not only be due to structural (ECM-based) changes in the heart. Explanted mouse and avian epicardium have been shown to stiffen during pre- and post-natal development stages [27, 28]. Stiffening also occurs as a result of remodelling in post-myocardial infarct tissue [29] and with age and disease in vasculature [30]. Finally, AFM has helped to resolve a stiffness disparity between juvenile and senescent-relaxed rat myocytes [26, 31]. This mechanical discrepancy was attributed to differences in myofibrillar content.

Although these results have advanced our understanding of myocyte mechanics, they have largely been limited to isolated myocytes *in vitro* (Fig. 3, left) or to *ex vivo* tissue explants lacking normal 'pre-stress', which is the tensile stress imparted by external entities *in vivo*. Experiments using isolated cultured cells can produce inconsistencies in mechanical data. For example, culture conditions such as substrate stiffness can drastically alter neonatal rat myocyte morphology, cytoarchitecture and ultimately, stiffness as indicated by force-indentation curves acquired by AFM [22] (Fig. 3C, left). Prolonged culture times can additionally increase myocyte stiffness during contraction [31]. Furthermore, while matching ECM cues such as

stiffness help to mimic the *in vivo* environment, these artificial approaches do not completely recapitulate it. To address this problem, *ex vivo* tissue explants are sometimes employed; however, discrepancies exist between stiffness reported for cultured cells *versus* explants. The latter sample type lacks the pre-stress typically created by adjacent tissue contracting against it, which can have profound effects on cells and even explants [29, 32]. Thus, unless pharmacological treatment establishes that measurements were indeed performed without pre-stress, partial contracture of the explant tissue may unknowingly skew data [31]. For the *in vitro* mechanical studies to more authoritatively offer insight on cardiac development, maturation and senescence, experimental conditions should rigorously approximate the physiological environment.

In light of the potential artefacts induced when cardiomyocytes are removed from their normal environment for *in vitro* studies, we have developed an *in situ* indentation method for the simple bilayered *Drosophila* heart tube (Fig. 3A and B, right). Due to its length scale, the fly heart tube is especially well-suited for indentation studies as it can be investigated anywhere longitudinally along its surface, removing the need for explantation or isolation (Fig. 3A, right). As the tube is unperturbed and is spontaneously beating only seconds before indentation, this method, to our knowledge, is the closest an AFM indentation experiment may come to an *in vivo* study of quantifiable single cardiomyocyte and sub-cellular stiffness. By indenting along the transverse axis of the tube, we are able to measure stiffness both at the midline where there is cell–cell contact (keeping in mind that the fly heart tube consists of bilateral rows of myocytes) and within the cell body. Before comparing cultured myocytes and heart tubes, it is important to note that one complication of *in situ* analysis is tissue geometry. For the fly specifically, a ventral layer of muscle covers the tube, and dissection methods cannot remove it without damaging the heart. Conventional mechanical analysis relies on the material being infinitely continuous [33], and corrections to account for layered materials have been limited to thin coatings with a high degree of stiffness mismatch [34, 35]. From the AFMs force-indentation plot, we can clearly observe differences in mechanical behaviour based on experimental conditions (Fig. 3C). At any given indentation depth (*x*-axis) into a sample, a greater reaction force response (*y*-axis) indicates a stiffer material (see arrows in Fig. 3C). As shown previously by Jacot *et al.* in rat, isolated neonatal mouse cardiomyocytes exhibited greater forces and were therefore stiffer when indented on glass coverslips as compared with those plated on a soft hydrogel tuned to ~8 kPa (Fig. 3C, left). In fly myocardium, we are able resolve differences in the force response at both shallow and deep indentation depths, which correspond to the ventral muscle and cardiomyocytes, respectively (Fig. 3C, right).

Both cultured myocytes and heart tubes can be probed with modest throughput, but a major benefit of *in situ* measurement is that intact tissues undergo comparatively little remodelling during characterization relative to cultured cells; while probing cultured cells, their motile nature makes repetitive probing near impossible. Given spatial variation in populations, this would make attempts to measure age-related differences in murine myocardium in the same way very time consuming and difficult. Therefore, we propose that *in situ* AFM-based analysis of the fly heart in conjunction with established myocardial-specific genetic manipulations can serve as a robust screening tool for the mechanical consequences of age and genetic modification of mammalian-conserved genes in cardiomyocytes.

Future perspectives: identifying changes with age-dependent diastolic dysfunction

While a plethora of techniques to monitor cardiac function and structure in flies have been developed, the methodology is based predominantly on optical analysis such that myocardial mechanics are therefore inferred [10–13]. Here, for the first time, we have developed the capacity to directly evaluate passive myocardial mechanical parameters, *in situ*, in *Drosophila*. These parameters play a significant role in dictating cardiac output in all organisms. Knowledge of the heart's material properties and how these change with age is central to developing effective therapies directed against age-associated diastolic dysfunction and diastolic heart failure. The ability to employ bioinformatics networking approaches, genome-wide screening techniques and genetic manipulation of any gene of interest in rapidly aging hearts generates enormous potential for high-throughput analysis of senescent-related changes in passive cardiac mechanics, a main determinant of diastolic function. Understanding the genetic basis of these changes should greatly expedite the development of effective treatments. Thus, our pioneering approach in flies will yield rapid and potentially translatable findings regarding cardiac aging and will facilitate the testing of novel mechanical models in higher organisms.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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