Title: In vivo measurement of muscle output in intact Drosophila

Article Type: Invited Article

Keywords: Optical beam transducer; jumping; insect muscle; shaking-B; amphiphysin; parkin

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Abstract: We describe our methods for analysing muscle function in a whole intact small insect, taking advantage of a simple flexible optical beam to produce an inexpensive transducer with wide application. We review our previous data measuring the response to a single action potential driven muscle twitch to explore jumping behaviour in Drosophila melanogaster. In the fruitfly, where the sophisticated and powerful genetic toolbox is being widely employed to investigate neuromuscular function, we further demonstrate the use of the apparatus to analyse in detail, within whole flies, neuronal and muscle mutations affecting activation of muscle contraction in the jump muscle. We have now extended the use of the apparatus to record the muscle forces during larval and other aspects of adult locomotion. The robustness, simplicity and versatility of the apparatus are key to these measurements.
Dear Sandy and Guy

Please find a revised manuscript, a supplementary movie, and our responses to the very helpful comments and suggestions from the reviewers.

best wishes

John
Responses to reviewers’ comments:

Ms. No.: METHODS-D-11-00108 Title: In vivo measurement of muscle output in intact Drosophila
Corresponding Author: Dr. John Sparrow Authors: Christopher J Elliott, DPhil; John Sparrow, DPhil

Reviewer #1:

1. In general, these authors should read through the manuscript carefully, checking punctuation and correspondence between text and figures (eg the various parts of Fig. 1). Done; all typos and inconsistencies highlighted by both reviewers have now addressed/corrected.

2. Page 2, abbreviations. The jump muscle is generally referred to as TTM in the literature - I would urge the authors to follow this convention to avoid confusion. Done, though we have left it as an abbreviation and in text we point out that in the Drosophila literature TDT has been/is used widely. GF - giant fibre neuron. Done.

4. Page 4, line 7 - large? What does this measure - shouldn't it be just sufficient? Removed from this instance but issue is addressed 'sufficient i.e. threshold' versus 'over-stimulation'. We have measured the effect of our routine stimulation parameters (28V, 1 ms) and are now explicit that these are ~10x threshold.

8. Page 8, line 25 - how were these parameters arrived at? We used these stimulus parameters as our collaborators (eg Allen and Murphey, Eur J Neurosci. 2007 July 1; 26(2): 439–445.) used "A pulse of 40–60 V for 0.03 ms from a Grass S48 stimulator", and 28V is the maximum our stimulator would produce. As indicated in the text, this provides a good safety margin for old as well as young flies.

9. Page 9, line 18 - rewrite, there are a number of different innexin proteins. Rewritten and extended slightly.

10. Page 10, line 11 - explain why this is interesting. The 'interesting' comment led to the discussion that followed in the same paragraph. This link is now made clear.

11. Page 10, first paragraph - ingenious, but an alternative explanation is that the GF in ShakB2 flies has a higher reliable stimulation threshold. We thank the referee for this suggestion and have mentioned it, though given the 10x greater than threshold stimulation we use we think this is not a likely explanation. Further experiments alluded to at the end of the paragraph will address this issue directly.

14. Page 13, line 7 - explain what the parkin mutation is. Done.

15. Page 13, lines 13/14 - they are not 'much reduced'. On reflection we agree – ‘much’ has been removed.

16. Page 15, lines 2/3 - the GF does not project from the CNS. Corrected.

17. Page 15, lines 17/18 - how do the authors know this? This was a text fragment from an earlier version where we posed it as a question to ourselves and was unfortunately not removed before submission.
18. Page 15, Fig 1 - why not also show a 6 leg WT take-off? We do, but not in same figure. Reference to another figure (Figure 3Ai)

19. Page 15, last line - measured on single legs? No. All amphiphysin measures were done on ‘6-leg’ flies. Now made clear.

20. Page 16, line 14 - turning the vertical adjustment on. We do not understand this comment in the context – we think it is because we wrote ‘turning the MM3 micromanipulator manually’ and this has led to a simple misunderstanding; to achieve greater clarity we have written ‘adjusting the MM3 micromanipulator manually’

21. Page 17, line 19 - explain why a force only half the weight of the fly is recorded. It is simply because the downdraught force will be sufficient to keep a fly flying against gravity. We cannot and did not expect to measure all this force i.e. it cannot be 100% efficient.

22. Fig. 6B - what are the vertical scales? Scales now added to figure

Reviewer #2: Major Comments

As this is a methods paper, the details of the device (components and construction/purchase) and experimental preparation (use) should be written such that the reader can perform the various experiments presented in the paper after either building or purchasing the device. As most, if not all, of the device specifications have been previously published, the authors should clearly reference where the details can be found and include any non-published relevant information in the Materials and methods (Section 2.0). As most users would likely purchase the device, including all the experimental preparation information in Section 2.0, even the previously published information, would create a single, comprehensive manuscript for those performing experiments using this novel apparatus. At a minimum, photographs should be added to the manuscript to better illustrate the experimental preparations. For instance, a close-up of Figure 2 should be included to show how the fly was mounted (location on fly where the fine needle is glued, etc.) and how the sharpened tungsten electrodes appear when inserted. A photo of the larval contraction measurements would also be very helpful.

In writing this article we were somewhat equivocal about whether we should put in, or not, all the details when, as we reference (but omitted to do so at the beginning of the Materials and Methods) the details that are comprehensively described in two previous papers from our research group. We agree with this reviewer’s comments and have made a number of substantial changes. First we have included a second photoimage (Figure 2B) which shows a close-up of the fly attached and positioned above the platform on the optical fibre. The stimulation electrodes are also clearly visible in this image. Second, we have included a lot more detail of materials and suppliers than previously and some more detail of how the experiments were done, and included a movie of the procedure. We hope this satisfies this request from this reviewer.

P4, 3rd paragraph - Include equation for Pythagoras’ theorem. If the vector of displacement is an important measure for this type of data, then this should be included in at least one of the figures. For instance, Figure 3 would be a good figure to include this information as Ci and Cii present peak vector displacement of the beam. We have inserted Pythagoras’ theorem, but also made it clear that in producing quantitative data we apply this to get the appropriate vector routinely.
P8, 2nd paragraph - What is the range of the short lengths of copper wire? Were these purchased already cut to standard lengths, and if so, where? We simply took copper wire from a standard piece used for making electrical connections, weighed a length to get a mass/unit length and used these. Individual weights of the copper pieces were also checked on a balance. The ‘source’ of the wire is now made explicit. We have also inserted information on the range of weights used to calibrate the apparatus and stated clearly that the weights were applied to the platform upon which the flies push.

P8, 3rd paragraph - Is MM3 a product or a company? Corrected to MM33 (product) and company inserted.

P9, 2nd paragraph - Provide details of small hook (material, size, etc.) and how to properly place the hook. Provide details of “short” cotton thread. A picture of this set up would be valuable. Details of the hook manufacture/size from an entomology pin (and source) now provided. Cotton (finest we could find) was of domestic origin. This is now made clear. We did not feel another photoimage was required. We have amended the text to provide more precision and altered the diagram to show more clearly the attachment of the larvae to the substrate (posterior) and through the hook (anterior) to the transducer. We do not consider a photoimage would add anything to this.

P11, 1st paragraph - Need to reference a paper that explains the standard geotaxis walking assay. Done

Figures - Please place the numbers indicating the size of the vertical bars vertically (as was done in Elliott et al., 2007). For instance, in Figure 1, Cii label the vertical bars as 50 µV. Labelling done vertically, as requested, in all figures

Figure 1 - Some of the light beam coming out of the flexible beam is missing the sensor, which I'm assuming doesn’t happen (true) with the apparatus as this would affect your results. Diagram adjusted. Labeling the top black arrow as "Nerve stimulation" or placing the red lightning bolt from Fig. 1A above the black arrows would be helpful. Thanks for the latter suggestion; implemented.

In order to see the differences in peak beam deflection between CS and shakB in Ci and Cii the vertical scale should be the same. Currently, the shakB vertical bar is much larger than CS instead of being the same size. Ciii should have the same vertical scale as Ci and Cii. Done

It would be interesting to see the wild-type output for 6 legs on the platform along with the shakB response (Ciii). We agree and appropriate data for comparison is shown in another Figure in the article. We have made clear cross referencing of this fact to allow the reader to make this comparison.

Question: How close does the flexible beam get to the mounted fly's thorax as the beam oscillates after the fly jumps? The upward motion of the beam looks quite large as though it could get close to or touch the fly mounted above. This is a good question and one that worried us once we started using the apparatus. The maximum displacement of ~500µm is less than the distance between the ventral surface of the fly thorax and the platform start position so the beam rebound does not directly impact on the underside of the fly, see Elliott et al 2007. We have done this by measurement (fly to beam before jump initiated and beam displacement as measured by the transducer) and direct observation. If it did impact the fly the time resolution of the transducer would adequately detect an
event or a truncation in the shape of the displacement peak. We have not addressed this issue in the rewrite, we would have done had the situation been the reverse.

Figure 2 - Add close-ups to show how the fly was mounted (location on fly where the fine needle is glued, etc.) and how the sharpened tungsten electrodes appear when inserted. As mentioned above – we have now done this.

Figure 3 - Should make vertical scale the same for all the plots in A and B so relative differences are readily apparent. Done

Why is the walking force of the amph NULL (Cii) the same magnitude of the jump performance of CS (Ci)? Figure 3 - Should make vertical scale the same for all the plots in A and B so relative differences are readily apparent. Now done for part A; but note that the units in A are µV but in B are in µN, so the data cannot easily be compared.

Why is the walking force of the amph NULL (Cii) the same magnitude of the jump performance of CS (Ci)? The force plotted in part B has been calibrated in µN, because the force is applied for a time longer than the time constant of the resonant beam. We have left the jump output in A in the raw form, µV, as the time scale is much faster than the time constant of the resonant beam, and so the actual forces applied cannot be calculated.

A vector of displacement plot, in addition to horizontal and vertical, would be useful for this data. We have done this. The output of the vertical vs horizontal plot was extremely messy and, so far, of no value in understanding what is going on. We have therefore chosen not to include it.

Figure 6 - The vertical scales for the wild-type and parkin mutant should be the same. The magnitudes of excursion of the parkin mutant looks larger than wild-type in B, but their magnitudes tend to be lower in Ci. Scales have been altered to being the same

Need to explain how larval velocity was measured. Now included in figure legend.
• We describe an apparatus for measuring muscle outputs from small intact insects.
• The apparatus is based on an optical fiber as a flexible beam transducer
• Transducer system developed to measure forces in jumping adult Drosophila.
• New applications to measure muscle outputs in walking, crawling and gripping
• A novel use to measure parameters of muscle contractions in larval crawling
In vivo measurement of muscle output in intact Drosophila

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Highlights: [highlights Elliott&Sparrow]
Abstract:

We describe our methods for analysing muscle function in a whole intact small insect, taking advantage of a simple flexible optical beam to produce an inexpensive transducer with wide application. We review our previous data measuring the response to a single action potential driven muscle twitch to explore jumping behaviour in *Drosophila melanogaster*. In the fruitfly, where the sophisticated and powerful genetic toolbox is being widely employed to investigate neuromuscular function, we further demonstrate the use of the apparatus to analyse in detail, within whole flies, neuronal and muscle mutations affecting activation of muscle contraction in the jump muscle. We have now extended the use of the apparatus to record the muscle forces during larval and other aspects of adult locomotion. The robustness, simplicity and versatility of the apparatus are key to these measurements.

Keywords: Optical beam transducer, jumping, insect muscle, insect behaviour, *shaking-B*, amphipysin, *parkin*, tergal trochanter muscle

Abbreviations:

CNS-central nervous system; GCI-giant commissural interneuron; GF-giant fibre (nerve); IFM-indirect flight muscle; LED-light emitting diode; PC-personal computer; TDT-tergal depressor of trochanter muscle; TTM–tergal trochanter muscle.
Introduction

Drosophila has become a major model genetic organism for the study of behaviour, neuronal and muscle function. A number of sophisticated technologies have been developed from those used on other animal systems to study neurophysiology and muscle function of a large number of mutants or transgenically expressed genetic modifications. These approaches include intra- and extra-cellular recording from nerve cells [24] or whole muscle fibres [2] and the study of muscle mechano-kinetics in so-called skinned fibres from the indirect flight muscle and the ‘jump’ muscle, the tergal-trochanter muscle, TTM (or sometimes, especially in the Drosophila literature, the tergal depressor of trochanter, TDT) [11, 21]. All these require sophisticated apparatus and dissection of the organism so may only approximate to in vivo physiological states and only indirectly relate to the behaviours exhibited by the whole organism, mutant or otherwise. Closing the gap between biological ‘preparations’ and the whole organism to study behavioural physiology as it relates to neuromuscular function is an important and interesting challenge. There have been a number of spectacular successes. In the Drosophila muscle field the undoubted success is the recording from single nerves and muscles of flying flies, as well as the forces they produce while behaving in ‘natural’ ways to environmental cues provided by the flight arena [13, 17, 18]. Such investigations require an extremely sophisticated and expensive set-up. Our approach has been one of simplicity, robustness and low cost.

1.1 Muscle output measurement in whole insects

In insects there are some classic examples for measuring muscle output in vivo in whole organisms, especially the jump muscle of locusts [6,10] and the stridulatory muscles of bush crickets [16]. For smaller insects these types of studies are much more difficult; relative functional estimates from jumping distances of Drosophila have been reported [27] but given the difficulties of measuring all the required parameters cannot lead directly to accurate estimates of jump muscle output.

We chose to study the jump muscle (TTM/TDT) of the adult fly which propels the fly by a single twitch contraction in response to a single action potential of the giant fibre (GF) (see [3] for a clear review). Flies jump as an escape response or to initiate flying. This response is easily evoked experimentally in a tethered fly by electrical
stimulation. The jump muscle is unusual in having no antagonistic muscle; the relaxed position is recovered due to the elasticity of the thoracic cuticle and the indirect flight muscles [12]. We describe here a simple, inexpensive transducer that allows us to measure the work done during single evoked jumps of a fly and, as we show in this report, muscle outputs from a number of other larval and adult locomotory behaviours.

1.2 The optical fibre transducer system

The basic principle has been to use an optical fibre as a flexible beam and to detect its displacement by measuring changes in position of the emitted light spot in one or two axes using a quadrant photodetector (Figure 1). The displacement vector is calculated from the two-axis data using Pythagoras’ theorem \(a^2+b^2 = c^2\) for the sides of a right-angled triangle, where \(c\) is the hypotenuse.

The flexible beam acts as spring. The position of the emitted light beam measures the displacement of the beam (specifically the tip of the fibre) due to the mechanical forces applied to it. The system does not impose any extra loads, including friction. The use of a quadrant photodetector and an optical fibre of suitable flexibility produces a very high sensitivity (typically 0.5 \(\mu V/\mu m\)) to applied deflections, and can readily be altered by choosing either other fibre optic materials or by changing the position of the fibre clamp (determines the functional beam length) or by varying the distance between the fibre tip and the photodetector to affect light spot size. The sensitivity and response frequency are limited by the resonant frequency of the beam.

As discussed previously [12] a major problem with using a flexible beam as a transducer is that its resonant properties can preclude us from directly measuring force. So when the period during which the fly applies forces to the beam is less than the resonant period of the beam we cannot measure force but only an ‘index of energy’ applied, which is related, though not in any simple direct manner, to muscle outputs. In any one set-up, with a constant beam with known resonant frequency this index provides a comparison of flies of different ages, genotypes etc. When the force is applied more slowly to the beam (i.e. application period > resonant period of the beam) we can measure force directly. Additionally, the ability of this system to allow a full range of muscle driven movements, rather than the isometric measurements
possible with stiff force transducers such as an Akers gauge, [27] allows us to 
measure muscle output during more natural behaviours.

1.3 Arrangement for jumping

The set-up for jumping experiments is based on the tethered fly being placed above a 
small platform affixed to the optical fibre (Figures 1A and 2). Each jump is activated 
by electrical stimulation of the giant fibre pathway using electrodes in each eye. Four 
typical vertical axis traces from a fly on the jumping setup (Figure 1Ci) show the 
high consistency of the responses. Repeated stimulation of the same wild type fly (up 
to 5 stimuli or more) does not cause a reduction in the mean vector displacement for a 
jump [15]. Between stimuli, the fly may move its legs, groom or even attempt to 
oviposit, so the starting position of traces may not be the same (see Figure 1Ci), but 
the waveforms are very similar and consistent.

1.4 Previous investigations of fly jumping using the apparatus

Our initial studies using this apparatus to investigate fly jumping led to a number of 
important conclusions about jumping behaviour, TTM function and the potential in 
using the jumping apparatus to study neuromuscular function in Drosophila with a 
variety of mutations.

By measuring the energy produced in a jump and that required to produce typical 
jumps made by Drosophila we showed 1) that no case needed to be made for 
mechanical energy storage prior to a jump in Drosophila unlike those needed for 
insects specialised for jumping such as fleas [7], locusts [6] and froghoppers [9]; 2) 
that as anticipated, the major part of the jumping force comes from the mesothoracic 
(middle) legs, the legs attached to the TTM, and that the pro- and meta-thoracic legs 
(front and rear) share two important but separate roles – the first to provide additional 
forces for jumping, the second to determine the forwards/backwards vector in the 
jump; 3) that in the absence of an antagonistic muscle to restore the relaxed TTM 
length this is achieved in part by the jump energy stored in the indirect flight muscles 
(IFM) as well as the thoracic cuticle. Another conclusion is that this work done by the 
TTM against the IFM is important in strain-activating the IFM to initiate flight[12].
We have shown that the apparatus can easily detect effects on TTM function and activation due to muscle mutations including the myosin heavy chain mutation, \( Mhc^5 \), and two troponin mutants (troponin I – \( hdp^2 \) and troponin-T - \( up^1 \)) [12].

In addition we have been able to measure jump muscle outputs to study mutations affecting synaptic transmission. Blocking the synthesis of octopamine, a catecholamine, using the \( M18 \) mutation of the tyrosine-\( \beta \)-hydroxylase gene (\( T\beta h \)) [19] significantly reduced (75%) jumping output [15], reinforcing the view that octopamine plays a major role in the flight component of the “flight or fight” response of Drosophila. Mutations of the \( \text{highwire} \) (\( hiw \)) gene affect growth and branching of the presynaptic terminals of the neuromuscular junctions. We found that this affects jump performance in young flies since five successive stimuli result in progressively smaller responses, suggesting a gradual decline in transmission or muscle fatigue that does not occur in control, wild-type flies [15].

1.5 Aims and objectives

Our initial goal was to develop a simple, robust and inexpensive apparatus that could be used with little training to measure with a medium to high throughput the jump muscle outputs of small intact insects, especially Drosophila. We have already achieved this with an apparatus based on using an optical fibre as a flexible beam transducer and demonstrated that it can provide useful quantitative physiological data from whole adult flies. Given the large range of available biochemical, behavioural, neuronal and muscle mutations that could potentially be of interest, measurements from a single fly should take only a short time and significant differences between genotypes and treatments should be obtainable from testing a relatively small number of individuals.

In this report we describe new data showing how the apparatus can be used to study the effects of mutations in genes affecting muscle membranes (T-tubules, \( \text{amph} \)) and the neural circuitry controlling the escape jump (\( \text{shak-B} \)). Importantly we have extended the use of the apparatus to determine novel muscle output parameters in adult and larval locomotion of both wild type and mutant individuals.

2 Materials and methods
The components, construction and use of the apparatus for measuring fly jumping have been described previously [15], so will only be described briefly here. The apparatus is shown diagrammatically Figure 1A and as two photoimages (Figure 2). Figure 2A shows the whole set-up, while Figure 2B shows a close-up of the tethered fly, held above the platform placed on the optical fibre beam. The two electrodes are inserted into the eyes to achieve electrical stimulation. An assembled version of the apparatus is available commercially from Digitimer, NL280 - XY Ergometer, (http://www.digitimer.com/neurolog/nl_transducers.htm).

The optical fibre (RS part 435-8202, RS Components Ltd, UK) is shortened to 160mm and clamped to an MM33 micromanipulator (Marzhauser-Wetzlar, Germany). A 5x5mm square of card is attached to the optical fibre 7.5mm from the movable end (see Figure 2B). A red light emitting LED is glued to the clamped end of the optical fibre and the light emitted at the movable end diverges onto a quadrant photodiode (RS part 652-027) mounted 0.5 to 1mm from this end of the light pipe. The signals from each quadrant of the photodetector are amplified by four current to voltage converters and input into a PC. Comparison of the four inputs is performed by DasyLab (measX GmbH & Co. KG, Moenchengladbach, Germany) to output the up/down and backwards/forwards movements as vertical and horizontal vectors. These are combined using Pythagoras’ theorem to produce the true vector values using Excel. Copies of our worksheets and supporting software are freely downloadable at http://biolpc22.york.ac.uk/drosophila/jumping/download

The system is calibrated by placing short precise lengths of a regular copper wire (standard electrical wire) onto the platform. The range of copper weights applied is 10-100mg. The beam displacements are linear up to 500µm with a slope of 0.5 µV/µm. This measured sensitivity is in the µN range. The linear response range spans beyond all the fibre displacements we have ever recorded from Drosophila.

For measurement/detection of flies jumping, walking, gripping the substrate or flying the flies, under carbon dioxide anaesthesia, are glued using Maribu Fixo Gum (Tamm, Germany) by their dorsal thoraces (see Figure 2B) to a 1 cm length of 0.2 mm tungsten wire (Agar Scientific) attached to a wooden cocktail stick. They are then positioned (Figure 2) using a micromanipulator (MM33,Marzhauser-Wetzlar, Germany) above the small card platform fixed onto the fibre optic (see Figure 2B).
For jumping the apparatus is adjusted until the beam spot is central on all four quadrants of the sensor. The positioning of the fly is important; all the legs need to be able to make contact with the platform and the distance between fly and platform affects the muscle output [12]. This is readily achieved, because flies gently lowered over the platform will reach out for and pull the beam towards them, then hold it at a fixed distance (See supplementary Movie).

Each jump is activated electrically by using tungsten electrodes (see Figure 2B), made from sharpened 0.7mm tungsten wire, to stimulate in each eye (28V, 1ms, ~10x threshold for a 3-day old wild-type fly). These are inserted by moving the electrode tip against each eye, causing a slight depression of the eye surface, and inserted by a gentle tap on the manipulator (See supplementary Movie). This electrical stimulation works well for routine investigations that involve activation of the jump muscles through the giant fibre.

Other behaviours are initiated spontaneously, such as (walking), or gripping the platform (Figure 2) by moving the platform downwards away from the fly using an MM33 micromanipulator or flying by blowing air from a position anterior to the fly.

For larval contraction measurements each third instar larva is pinned through its posterior end with a fine tungsten dissection pin to polymerised Sylgard (Dow Corning, USA) in a 5cm Petri dish and a hook, made by bending a small entomology pin (#E6871, 0.14mm diameter, 10mm long, Watkins & Doncaster, UK) into a right angle, placed through the mouthparts (Figure 6). A short cotton thread (the finest sewing cotton we could obtain) between hook and flexible beam is pulled gently taut and the position of the larva is adjusted so the thread is vertical. The midpoint of the contractile movements has to be when the beam illuminates all 4 quadrants equally; to achieve this we move the Sylgard dish to which the larva is attached rather than the sensor beam.

3. Results

We have now extended our investigations to study aspects of the activation pathway of jump muscle contraction from the CNS to the post-synaptic events in the muscle itself, to other adult behaviours (gripping the substrate, walking and flying) and to measure the forces of peristaltic larval crawling movements.
3.1 Gap junctions and the GF activation of jumping

The shak-B^{2} mutation occurs in the shaking-B gene, one of five innexin encoding genes in Drosophila. Innexins are a group of related proteins found in other invertebrates that are an integral part of gap junctions [22] and the shak-B^{2} mutation prevents the formation of electrical synapse in the Drosophila giant fibre system [24]. Our analysis of this mutation (Figure 1 C-D) demonstrates how the jumping apparatus permits a detailed analysis of subtle behavioural effects within the whole organism. It was previously known from physiological recordings of dissected flies and anatomical tracer studies that the left and right GF neurons are linked by the GCI neurons in the brain, using homotypic gap junctions (see Figure 1B), while each GF is linked to the TTM motoneuron via a mixed chemical and electrical synapse [1, 23, 24]. The importance of each component in escape behaviour is not known nor is the relative role of the left/right GFs clear. Do the GCIs couple the GFs so tightly that both left and right TDTs always function simultaneously and the firing one GF is sufficient for a full jump?

Flies with viable mutations of the shak-B gene fail to jump in response to a ‘light-off’ stimulus [4] due to defects in the GF pathway. The four force traces (Figure 1Ci) show typical responses and their reproducibility. Studying shak-B^{2} flies with just one mesothoracic leg in contact with the beam shows that some stimuli are followed by a jump but other identical stimuli give no response at all; the waveform is all or nothing (Figure 1Cii) which is not the case for wild type (Figure 1Ci). About 50% of young (3-day old) shak-B^{2} flies with 6 legs on the platform fail to jump (Figure 1Di). In the others, the trace is very variable (trace from one fly shown, Figure 1Ciii) compared to wild type jumping with 6 legs (Figure 3Ai) and interestingly (see discussion below) many of the shak-B^{2} traces start with an upward rather than downward trace. Detection of this phenomenon is an advantage of a sensor system which records in multidimensional space. Those flies that do jump produce less than 50% of the jump output of wild-type flies (Figure 1Dii). Together the data suggest that in the shak-B^{2} flies only a single mesothoracic leg (left or right) is working. This could be due to failure of either the coupling of GFs in the brain, or to coupling of the GFs to the TTM motoneuron, both physiologically affected by the shak-B^{2} mutation [23]. We suggest that this results from the gap junctions between GFs and GCIs in the brain.
failing in the \textit{shak-B}^2 mutants, as the cholinergic coupling between GF and TTM motoneuron is enough to maintain the downstream circuit [1]. This is direct evidence for a tight functional coupling of the GFs in the freely escaping fly. We also propose that the GFs make easily modulated, polysynaptic connections with the motoneurons for the pro- and metathoracic leg muscles, resulting in these legs being drawn up close to the body, so reducing the drag during flight. However, these chemical connections seem more robust than the electrical GF to TDT motoneuron connection. An alternative explanation (for which we thank one of the reviewers) is that the \textit{shak-B}^2 system has a higher stimulation threshold to produce a reliable jump output; but, as we give \textasciitilde{}10x the threshold stimulus, we think this is unlikely. Further experiments using the jump apparatus and other techniques will be required to resolve these proposals, but without the apparatus these effects in single flies are unlikely to have been detected.

3.2 T-tubule function in jump muscle activation

Amphiphysin is a widespread protein occurring in specialised membrane locations within different tissues, including epithelial and neural cell types. In Drosophila it occurs post-synaptically at the neuromuscular junctions and is required for the localization of several post-synaptic proteins, but not directly for synaptic vesicle reception [26]. Null mutations of the single Drosophila amphiphysin (\textit{amph}) gene are however viable and exhibit no major effects in larva; adults behave completely normally except that they are flightless [25]. This phenotype was correlated with severe disturbances of the extensive reticulated T-tubules of the IFM [25]. Since T-tubules transmit the electrical changes of the sarcolemma into the close proximity of the sarcoplasmic reticulum and the myofibrils, these observations showed a highly organised T-tubule pattern is important for the rapid activation of the IFM for flight. We asked whether the TTM, with its very extensive neural arborisation was also sensitive to the absence of amphiphysin. The \textit{amph}^{26} null flies (Figure 3Aii) using all six legs produce only a minimal jump energy compared to wild type controls (Figure 3Ai, 3Aii and 3Ci) which will prevent flight initiation and may supersede the IFM deficits in generating the \textit{amph}^{26} null flightless phenotype. Observations of tethered flies, prepared for jump testing, show rhythmic movements of the legs, leading to low amplitude beam displacements suggesting they are attempting to walk (see
Supplementary Movie). In \textit{amph}\textsuperscript{26} flies this walking behaviour in terms of the maximum beam displacement (equivalent to ‘walking force’) is not significantly different from the wild type controls (Figure Cii). We confirmed that their maximum walking speed is normal using a standard geotaxis walking assay \cite{20} in a vertical column (Figure 4Ciii). Thus our data shows a significant difference in the importance of this protein between the jump muscle and other leg muscles.

3.3 Adult walking and substrate gripping forces

A key factor in jumping and walking is that each leg makes good contact with substrate, especially important when a fly walks vertically or upside down. When mounted for jumping, some flies spontaneously make leg movements, raising or lowering their legs, or attempting to walk. These relatively small movements are well within the sensitivity range of the optical beam apparatus (Figure 3B) and as we can resolve movements/forces in the vertical axes it is possible to resolve walking (horizontal movements) from leg retraction/extension movements (vertical axis) and the forces produced. This type of behaviour is rather erratic. Changes in the set-up and analytical methods need developing further to produce useful data on wild-type and mutant leg movements. Gripping the substrate (possibly different substrates) is a behaviour that we and others have previously ignored as part of take-off/jumping.

We have obtained data (Figure 4) on the ability of flies to grip the substrate by setting up flies to jump and then gradually raising them away from the platform using the MM33 micromanipulator to which they are attached (Figure 2). The vertical trace (Figure 4) shows that the fly hangs on to the substrate with increasing force until a point is reached when it begins to let go. This is apparent as a sudden drop in force followed by some resonance from the beam. It is clear that the fly does not let go with all its legs at once as there are at least two rapid drops in force.

3.4 Detecting forces produced by a fly in flight

The apparatus is also sensitive enough to detect air movements below a flying fly by the mean displacement of the beam (Figure 5). The vertical and horizontal outputs are oscillatory (Figure 6), but are both well below the ~200Hz of the Drosophila wingbeat, being 14.95 ± 3.38 and 10.88 ± 3.05 Hz respectively. The oscillations detected are likely to be dominated by the resonant properties of the beam; the
different frequencies of vertical and horizontal beam tip displacements probably indicate a complex movement. The apparatus can thus be developed to measure the force and the position of the downdraughts from a flying tethered fly. At the end of flying the upward output (vertical axis trace) reflects the fly grabbing the platform and retracting its legs (Figure 5). This is a similar response to that in which the fly is moved vertically while gripping the platform (Figure 4).

3.5 Measuring muscle contraction in whole larval peristalsis.

The properties of the optical beam transducer, particularly its time/force sensitivity, are well suited for measuring slow movements such as those exhibited by peristaltic larval bodywall muscle contractions responsible for larval crawling. Locomotion of larvae homozygous for the parkin$^{25}$ null mutation [14] had been shown to be slow (Vincent et al, submitted). Attaching wild type and parkin$^{25}$ third instar larvae to the apparatus (Figure 6A) shows that both genotypes produce rhythmic forces from these contractions (Figure 6B) which can be measured and persist over an extended period. Analysis of these rhythmic outputs show that compared to the wild type controls the parkin larvae produce contractions of similar amplitude but significantly lower contraction frequencies and reduced forces, a form of bradykinesia. Thus with this simple apparatus the ‘slow’ locomotion can be readily analysed in detail. This novel application of the apparatus promises to be particularly useful, especially as the larval Drosophila bodywall muscles have become a well developed model system for electrophysiological studies of neural mutations [5,8].

4. Discussion/Conclusion

We have described a simple, inexpensive apparatus that has been used primarily and successfully to investigate the forces produced during jumping of intact Drosophila. It provides a relatively direct measurement of the contraction of the jump muscle (TTM) that we have shown can be used to investigate detailed aspects of jumping behaviour. This is readily applicable to other small insects. The data variance is such that for most experiments we need test no more than 15-20 individuals of each genotype, age or treatment and individual fly assessments take only 2-3 min.

In Drosophila measurements of jumping behaviour and force production in neuronal and muscle mutations reveal the utility of the apparatus in investigating the details,
especially the mechanical outputs, of aspects of the activation of jumping, from the CNS to the muscle, the neuromuscular junction and within the muscle itself.

We have shown the ready adaptability of the system to measure other behavioural and muscle outputs. The slow contraction of the larval bodywall muscles, used for crawling, are especially suited to the dynamics of this set-up. The data obtained permit measurements of the oscillatory pattern generator in the CNS, the amplitude of the forces produced, and the kinetics of the relaxation/contraction cycles of these muscles.

Further applications of this apparatus can address the integrated function of many muscle groups. Thus we have indicated with preliminary data that investigations of fly walking, their ability to grip and exert forces on their substrate, and of the downdraft forces of flying flies are all possible. While we expect this simple apparatus to find wide application in investigating Drosophila mutants, it is also readily applicable to the study of other small insects.

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Figure Legends:

Figure 1. Flexible beam recordings show behavioural consequences on jumping of shak-B^2, a neuronal mutation. A. Schematic of the apparatus, showing the way the LED beam projects across the gap between the end of the flexible beam and the optical sensor which contains 4 photodiodes. Comparison of the intensity across all 4 diodes provides a two dimensional projection of the movement of the fly. B. Diagram of giant fibre (GF) projecting from the CNS to the TTM motor neuron (TTMmn), which projects to the tergal trochanter (TTM) muscle. Both GFs are linked by gap junctions to the giant commissural interneuron (GCI) within the CNS. Each GF makes an electrical+chemical synapse with the TDT motoneuron. The shak-B^2 mutation increases the probability of failure at the GF → motoneuron and GF -> GCI
synapses. In a wild-type (Canton-S; CS) fly with only 1 mesothoracic leg free to stand
on the platform each stimulus leads to a consistent ergometer output (Ci), but the
response of a shak-B^{2} fly to a series of stimuli is all or nothing (Cii). In wild type flies
free to place all 6 legs on the platform, the output usually starts with a consistent
downwards deflection , (Figure 3A). However, in this sample shak-B^{2} fly (Ciii) only
1 trace has an initial downward deflection; most of the stimuli lead to an upwards
response with very variable latency and amplitude. D. Quantification of the response.
(Di) only 50% of shak-B^{2} flies show a jump response, and in those that do, the
response (Dii) is about half the wild-type. One explanation for this is that the response is
unilateral with only the left or the right jump muscle active. Arrow (Ci-iii) indicates
time of stimulation; horizontal recordings not shown. ** and *** (Di & Dii) show
statistical significance at P<0.01 and 0.001 respectively.

Figure 2. Layout of the apparatus to measure jump muscle output. A. Photograph,
from above, shows position of flexible optical fibre beam, with small platform at one
end. Fly is mounted on a tungsten pin, so as to rest its leg(s) on the platform. Beam
projects onto quadrant photodiode, aligned so intersection of the 4 diodes is on the
beam axis. Two sharpened tungsten needles are positioned at an angle of 45 ° to
impale the eyes for electrical stimulation of GF pathway. B. Close-up photograph of
the fly resting on the platform, with the stimulating electrodes ready to be inserted
into the eyes. This is one frame from the Supplementary Movie, which shows the
process of recording.

Figure 3. Flexible beam recordings allow comparison of jumping and walking
movements and forces. Recordings of four wild-type (CS) fly jumps (Ai) using all 6
legs. Note consistency of the downwards force and greater variability of horizontal
forces. In the amphiphasin (amph) null (Ai), the combined jump muscle and leg
muscle output (six legs on platform) is very small, and similar in magnitude to the
noise in the recordings. B. Before the electrodes are inserted, flies on the platform will
try to walk deflecting the platform and beam; sometimes they release the platform, so
that trace jumps backwards at intervals. This is also seen in the Supplementary Movie.
Measurement of the peak-peak deflection of the trace provides an indication of the
force that the fly can generate as it walks, in this case ~100 μN. C. Quantification of
the jump muscle output (Ci) shows that the amph^{26} null releases minimal energy from
the TTM, but the walking force (Cii) (generated by the same flies) does not differ between wild-type and the *amph* null. This was confirmed in a conventional vertical climbing assay (Ciii). Arrows in Panel A indicate time of stimulation.

Figure 4. Flexible beam recordings permit measurement of the adhesion force by a wild type fly. A. Recording of the force as the fly is gently lifted away from the platform by turning the MM3 micromanipulator (Figure 2) manually.

Figure 5. Flexible beam detects the downdraft from flying flies. The vertical and horizontal traces show that as the platform is moved towards a flying tethered fly the beam platform detects the downforce (oscillatory trace segments). The mean force (about 5 µN; half the weight of a fly) exerted is seen as the displacement of the beam from its original position. The upward movement (vertical trace) at the completion of flying reflects the fly gripping the platform and pulling it towards itself by contracting its legs.

Figure 6. Flexible beam acts as a sensitive tension transducer for larval contraction. A. Larva pinned to a Sylgard dish with small hook inserted through its mouthparts. Short cotton thread between hook and flexible beam is pulled gently taut and larva position adjusted so thread is vertical. B. The peristaltic contractions have sufficient force to move the beam. As their frequency is below the resonant frequency of the beam, a direct measure of the force is produced. Force traces from peristaltic contractions of *parkin* and wild-type (CS) larvae. C. Quantitative analysis shows *parkin* and wild-type larvae have the same peak-peak amplitude (force) of the contractions (Ci) but the *parkin* larvae contract less frequently (Cii). D. Slower free locomotion of *parkin* larvae on an agar surface.

Supplementary Movie: A recording (with descriptive commentary) of the mounted fly being brought up to the platform, the fly grabbing and 'walking' and grooming on the platform, the insertion of the electrodes and stimulation through the electrodes. Each stimulus is indicated on the sound track by a quiet click from the stimulator. At this frame rate (original is at 15 frames/s) the leg extension, beam movement and wing-flick are hard to resolve.
References


Figure

A

LED

flexible beam

tungsten pin

sensor

Ci

CS one leg

shak-B one leg

Ciii

shak-B six legs

Di

% flies jumping

Dii

peak beam deflection (µm, mean ± SE)

CS (9/9)  

CS x shak-B (11/11)  

shak-B (8/13)  

**  

***
A flexible beam

stimulating tungsten electrodes mounted in MM33 manipulators

tungsten pin holding fly

MM33 manipulator mounts for fly and sensor

B
Figure

Ai

Ver

CS

Hor

amph^26 NULL

Aii

Ver

amph^26 NULL

Hor

50 ms

B

Ver

CS

Hor

1 s

Ci - jump performance

***

NS

Cii - walking force

NS

Ciii - walking speed

NS
fly moved upwards
fly gradually brought closer to beam
fly flying close to beam
fly landed

Ver

Hor

20 μN

1s
A flexible beam LED sensor

B

wild-type

parkin

C

Ci

force (µN, mean ± SE)

wild-type parkin

Cii

contractions / minute (mean ± SE)

wild-type parkin

D

larval velocity (mm/s, mean ± SE)

wild-type parkin

Figure
Supplementary Material

Click here to download Supplementary Material: Elliott_Sparrow.mov