Imaging Modalities for Semi-Translucent Animals and Their Impact on Quantitative Analysis

Benjamin Risse*, Dimitri Berh*, Nils Otto†, Xiaoyi Jiang* and Christian Klämbt†
*Departement of Computer Science, University of Münster, Germany
†Institute of Neuro and Behavioural Biology, University of Münster, Germany

***Abstract*—Imaging and analyzing the locomotion of semi-translucent animals such as Drosophila larvae or C. elegans has become an integral subject of biological research. However, acquiring contrast rich and high resolution measurements is still challenging. Several new imaging modalities have been introduced recently, ranging from optical to non-optical acquisition techniques. Here we briefly survey the modalities by grouping them into methodological clusters and discussing their challenges such as field-of-view, signal-to-noise ratio and other biological needs like the integration of stimuli. Especially the relation between imaging and subsequent behavioral analysis is examined. We focus on the new FTIR-based FIM technique, which is used to detect the contact surface between the arena and the animals. FIM facilitates high-contrast and high-throughput behavioral analysis by eliminating many inaccuracies on a physical level.**

I. Introduction

The quantitative analysis of behavior has become an integral subject of biological research. For example, the relationship between genes and behavior or behavioral neuroscience has become increasingly informative over the last years [1], [2]. Central model organisms to study genetics or neurobiological bases of behavior are *Drosophila melanogaster* larvae [3], *Caenorhabditis elegans* worms [4], [5] or planarians such as *Schmidtea mediterranea* [6]. All mentioned animals have in common, that there are a plethora of molecular tools available for both, manipulation and imaging. Furthermore, these animals are easy to culture in laboratory environments so that large numbers of animals can be screened to increase the statistical strength.

Over the past decade there was an exponential growth of automatic behavioral analysis methods including both, imaging modalities (i.e. the image acquisition hardware) and subsequent quantifications (i.e. the software) [7], [8], [9], [10]. A precise phenotypical quantification however requires appropriate image acquisition. Therefore, much effort has been invested into optimizing the acquisition [11], [12], [13].

The contribution of this extended abstract is a brief survey covering recent imaging techniques by identifying several methodological groups, each offering specific advantages and disadvantages. Furthermore, the impact of imaging onto subsequent quantification is discussed using this methodology in terms of behavioral analysis. In particular, we compare the newly developed FIM setup (Figure 1) with other modalities and discuss its possibilities.

II. Imaging Modalities And Its Challenges

Imaging small animals with good spatial and temporal resolution is still a challenging task [14]. On the one hand, high resolution and good contrast is necessary to extract complex movement features like the body bending angle or peristaltic movement [15], [16]. On the other hand, a large field-of-view is desirable to enable high-throughput screening [17]. In addition, the semi-translucent character of the animals aggravates faithful imaging [13]. To improve the experimental conditions a moist tracking surface (e.g. agar) is often used leading to disturbing artifacts such as light reflections [18].

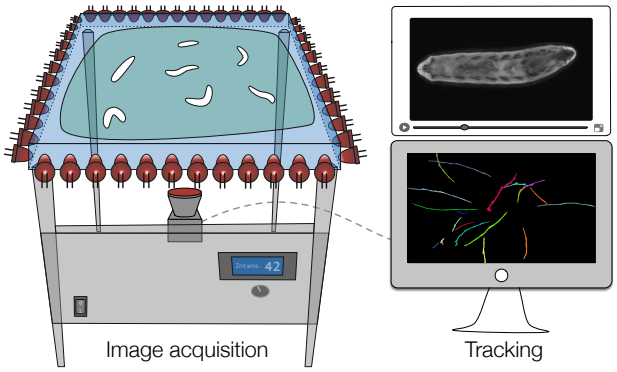

Fig. 1. Relation between image acquisition and tracking in the FIM setup: The FIM imaging modality is sketched on the left. A resultant raw image and several trajectories are given on the right. For more details see Section II-A4.

Therefore the two central challenges of imaging are the throughput (mainly limited by the field-of-view) [19] and the foreground-background contrast (mainly limited by the size and unsuitable appearance of the animals) [13]. Unfortunately, increasing the field-of-view will inevitably reduce the resolution of the animals and thus aggravate faithful segmentation because of a decreasing signal-to-noise ratio (and vice versa) [15]. Thus, only imaging modalities with a very high signal-to-noise ratio enable comparatively large field-of-views: An increase of the field-of-view indeed causes a quadratic decrease in the domain of the extractable features, but given sharp and noise-free detections the overall precision is maintained. Furthermore, the integration of stimuli (e.g. light [20], odor [21], temperature [15]) or the integration of optogenetic techniques [22] requires flexible conditions [12].

These challenges are addressed in many publications covering different imaging modalities to improve the measurements. Current research interests are mostly focused on the following challenges:

- Field-of-view vs. resolution of a single animal (i.e. high-throughput vs. precise feature extraction)
- Signal-to-noise ratio (i.e. high contrast without noise or disturbing artifacts)
- Integration of stimuli
- Generalizability and range of applications

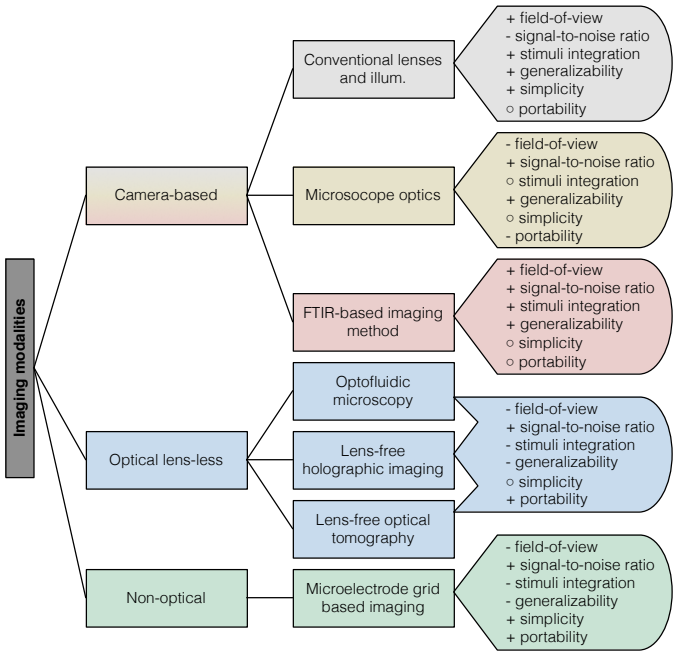

Fig. 2. Overview of the imaging modalities. Advantages and disadvantages are roughly classified according to the list given in the text (+: optimized, –: not optimized, ○: semi-optimal). Color code corresponds to Figure 3.

- Simplicity (in terms of construction and usability)
- Portability of the setup

A. Imaging Modalities Overview

Imaging modalities range from camera-based setups over optical but lens-less setups (i.e. using a sensor but no lens) to non-optical setups (i.e. using no imaging sensor at all).

1) Camera-based Imaging Techniques: A common imaging technique bases on a camera with an appropriate lens and lightning conditions to image animals in an open-field arena. Camera-based imaging can be separated into setups using different optics and illumination strategies. The main difference in optics is related to the magnification, so that the two major subgroups either use microscopes [23], [24], [18], [16], [25] or conventional lenses [15], [17], [26]. Illumination can be done by incident light [27] or transmitted light [26], in which the latter strategy is often used in microscope setups (i.e. bright-field illumination [17]).

Imaging With Conventional Lenses: Multiple setups using conventional lenses have been introduced in the past, ranging from single-animal to multi-animal imaging [15], [17], [26]. To increase the throughput but still enabling high signal-to-noise ratios, multi-camera and multi-well setups have been introduced [7], [6]. In a different approach, dark-field-illumination was utilized as a more elaborate illumination strategy [28]. However, if a very high precision (resolution per animal) is necessary, conventional lens systems still lag behind the microscope setups.

Imaging Using Microscopes: Microscope-based setups use microscope optics in combination with an appropriate camera [29], [23], [24], [18], [16], [25]. These setups are very popular because they offer a very high spatial resolution of the animals so that artifacts, noise or semi-optimal illumination conditions can be compensated to a certain degree.

2) Optical Lens-Less Imaging Modalities: As an alternative to camera-based imaging, optical but lens-less modalities have been introduced. While still using image sensor arrays (i.e. CMOS/CCD), no optics are used at all. Examples are the microfluidics-based lens-less on-chip optical imaging (called optofluidic microscopy) [10], [30], the lens-free holographic imaging [9] or the lens-free optical tomography imaging [31].

3) Non-Optical Imaging Technique: Beside optical imaging techniques, non-optical modalities for phenotypic characterization have been introduced recently. This is done by lens-less and image-sensor-less posture and motion characterization using a grid of micro electrodes to measure the electrical resistance allover the grid [32].

4) FTIR-Based Imaging Method: The underlying physical principle of the above described non-optical modality is also used in touch-screens, to measure the position of a touch on LCD displays. Similarly, optical principles such as frustrated total internal reflection (FTIR) are used to measure refracted light caused by physical touches on an illuminated but transparent plate [33]. This technique has been adapted for behavioral experiments recently (called FTIR-based Imaging Method; FIM) [8]. In the FIM setup, the measurement surface (an acrylic glass plate) is illuminated by infra-red LEDs mounted at the edges of the plate (Figure 1). Because of total internal reflection, light is completely reflected inside the acrylic glass. Only light reflections caused by the animals crawling on the surface medium (e.g. agar) are detected by a camera, which is mounted underneath. This technique has been successfully used to image Drosophila larvae, C. elegans, planaria or Arabidopsis roots [8].

B. Comparison Of The Different Techniques

As illustrated in Figure 2, each modality has its advantages and disadvantages. Camera-based imaging with conventional lenses usually offers a large field-of-view and the highest generalizability and simplicity so that the integration of different stimuli is possible [19], [28]. However, the signal-to-noise ratio is far behind the possibilities using microscope optics, especially if subtle details of small animals need to be assessed [23]. Thus microscopes are still used for precise imaging of Drosophila larvae, C. elegans or planarian in many locomotion studies [29], [18]. However, the limited field-of-view of microscope-based setups limits the size of the arena in which the animal can move. Even though this can be addressed by a movable camera [18], [16] or tracking stage [29], [23], [24], [25], these setups are still limited to a single animal, since only one animal can be followed over time.

Optical but lens-less imaging techniques have in common, that they offer a very high and detailed spatial resolution of the objects without suffering from the aforementioned contrast limitations [9], [10], [30] (even in 3D based on tomograms [31]). On the other hand, it is impossible to record moving animals since the temporal resolution as well as physical limitations prevent these techniques from acquiring movement given feasible frame rates.

In contrast to optical lens-less imaging modalities, the non-optical imaging technique is able to record a moving

animal [32]. While the spatial and temporal resolution is feasible to track a freely moving C. elegans worm, both, the field-of-view and the per-animal-resolution is limited by the electrode grid, which will always lag behind the physical possibilities of optical methods. On the other hand both, lens-less and non-optical techniques are designed to be compact and portable [32], [30].

The FIM setup incorporates the large field-of-view of camera-based techniques with the sensitivity of non-optical techniques since only touches of the animals on the measurement surface are recorded by a camera without disturbing background artifacts [8]. Thus, high signal-to-noise ratios are achieved for crawling but semi-translucent animals. Furthermore, given the high contrast internal structures are imaged without utilizing microscope optics [34], [8]. Since the camera is mounted underneath, the integration of external stimuli is done easily.

III. Quantitative Phenotyping

For quantification, the acquired images need to be analyzed using image analysis algorithms to generate shape and locomotion related features [35]. Of course, the precision and reliability of all subsequently calculated features strongly depends on the quality of the imaging modality. In addition, the range of desired features can differ according to the biological question. Current quantification techniques are mostly focused on the following trade-offs:

- Shape quantification vs. tracking
- Single- vs. Multi-target-tracking
- Point- vs. Contour-tracking
- Stimulation integrated in the tracking software
- Inner Structures used during quantification

The quantitative readout can be restricted to the size or shape of the animals [9], [10] or can be done over time to track the locomotion (i.e. tracking) [8], [32], [28], [25]. Locomotion related features can be calculated from the animals center [7] (i.e. point-tracking) or include posture features calculated from the animals contour [15] (i.e. contour-tracking) [35]. Furthermore, tracking can be differentiated into precise single-target tracking approaches to maximize the computable locomotion features [29] or into multi-target tracking to increase the throughput [26].

In addition, several specific questions can be addressed by integrating stimuli such as heat [19], light [8], airborne cues [28] or optogenetics [22] into the quantification. For example, if the locomotion needs to be analyzed on the level of inner structures like muscles, directed GFP expression is used in combination with microscope-based setups [36], [37].

A. The Impact Of Imaging On Quantitative Phenotyping

As illustrated in Figure 3, different imaging modalities lead to different trade-offs in the subsequent quantitative analysis. Analogous to the field-of-view, multi-target tracking is only possible using non-macro optics [28], [17]. On the other hand, a more detailed analysis of shape-related locomotion is possible using microscope-based setups but limits the throughput

Imaging modality	General readouts	Tracking readouts
Conventional lenses and illum.	Tracking	Single- & **Multi-target**
		Point & Contour
	Stimuli integration	
Microsocope optics	Shape quantification	
	Tracking	**Single-target**
		Point & **Contour**
	Stimuli integration	
	Inner structures	
FTIR-based imaging method	Shape quantification	
	Tracking	**Multi-target**
		Contour
	Stimuli integration	
	Inner structures	
Optical lens-less	Shape quantification	
	Inner structures	
Non-optical	Tracking	**Single-target**
		Contour

Fig. 3. Overview of the phenotyping possibilities. Quantitative readouts are assigned to each imaging modality. Color code corresponds to Figure 2. Common tracking methods for each imaging modality are highlighted in bold.

to a single animal at a time [29], [25]. For this reason, point-tracking algorithms are frequently used in setups utilizing conventional lenses, while microscope setups almost always characterize the locomotion based on the animals contour [7], [29], [26].

All above mentioned optical but lens-less modalities are restricted to shape related phenotyping only, since these techniques are not able to generate motion related measurements [9], [10], [30]. However, given the very high spatial resolution, subtle structures can be measured even in three dimensions [31].

The microelectrode grid based non-optical imaging uses tracking software initially programmed for microscope-based setups [32]. Thus, the software calculates the contour from a matrix generated by resistance changes on the grid. However, tracking more than one animal at a time can hardly be realized given nearby animals.

Tracking animals using the FIM setup benefits from the high signal-to-noise ratio: Image processing is reduced to a minimum, while the animals maintain a very sharp appearance [8]. Thus, changes in the animals area can be used to quantify peristalsis, even if the resolution of the animals is below 25 pixels larval length [38]. Furthermore, internal organs like the trachea or structures like gaps between the muscle fibers were used to quantify locomotion in a more precise manner [34]. In addition, tracking is not disturbed by outer stimulations since only the touches of the animals are measured.

IV. Conclusion And Discussion

Here we compared several imaging modalities for small and semi-translucent animals in terms of their impact on subsequent quantitative analysis. In particular, we examined these modalities based on their throughput, range of extractable features and specific biological needs with special focus on the new FIM method.

Since the physical principles of light are almost unlimited in terms of spatial and temporal resolution, limitations are only given by the resolution of the camera. Fortunately, modern cameras are both inexpensive and high-resolution. It became apparent that FIM combines the strengths of camera-based and non-optical imaging to maximize the signal-to-noise ratio (i.e. sensitivity) so that quantitative analysis strongly benefits from this imaging modality.

REFERENCES

[1] M. B. Sokolowski, "Drosophila: Genetics meets behaviour," *Nat. Rev. Genet.*, vol. 2, no. 11, pp. 879–890, 2001.

[2] A. Leshner and D. W. Pfaff, "Quantification of behavior." *Proc. Natl. Acad. Sci. U.S.A.*, vol. 108 Suppl 3, no. Supplement 3, pp. 15 537–15 541, 2011.

[3] J. T. Vogelstein, Y. Park, T. Ohyama, R. Kerr, J. W. Truman, C. E. Priebe, and M. Zlatic, "Discovery of Brainwide Neural-Behavioral Maps via Multiscale Unsupervised Structure Learning." *Science*, p. 1250298, 2014.

[4] A. E. X. Brown, E. I. Yemini, L. J. Grundy, T. Jucikas, and W. R. Schafer, "A dictionary of behavioral motifs reveals clusters of genes affecting Caenorhabditis elegans locomotion." *Proc. Natl. Acad. Sci. U.S.A.*, vol. 110, no. 2, pp. 791–796, 2013.

[5] N. Roussel, C. A. Morton, F. P. Finger, and B. Roysam, "A Computational Model for C. elegans Locomotory Behavior: Application to Multiworm Tracking," *IEEE Trans. Biomed. Eng.*, vol. 54, no. 10, pp. 1786–1797, 2007.

[6] D. Blackiston, T. Shomrat, C. L. Nicolas, C. Granata, and M. Levin, "A Second-Generation Device for Automated Training and Quantitative Behavior Analyses of Molecularly-Tractable Model Organisms," *PloS one*, vol. 5, no. 12, p. e14370, 2010.

[7] C.-C. J. Yu, D. M. Raizen, and C. Fang-Yen, "Multi-well imaging of development and behavior in Caenorhabditis elegans," *J Neurosci Methods*, vol. 223, pp. 35–39, 2014.

[8] B. Risse, S. Thomas, N. Otto, T. Löpmeier, D. Valkov, X. Jiang, and C. Klämbt, "FIM, a Novel FTIR-Based Imaging Method for High Throughput Locomotion Analysis," *PloS one*, vol. 8, no. 1, p. e53963, 2013.

[9] S. O. Isikman, I. Sencan, O. Mudanyali, W. Bishara, C. Oztoprak, and A. Ozcan, "Color and monochrome lensless on-chip imaging of Caenorhabditis elegans over a wide field-of-view," *Lab Chip*, vol. 10, no. 9, pp. 1109–1112, 2010.

[10] X. Cui, L. M. Lee, X. Heng, W. Zhong, P. W. Sternberg, D. Psaltis, and C. Yang, "Lensless high-resolution on-chip optofluidic microscopes for Caenorhabditis elegans and cell imaging," *Proc. Natl. Acad. Sci. U.S.A.*, vol. 105, no. 31, pp. 10 670–10 675, 2008.

[11] C. Sinadinos, C. M. Cowan, A. Wyttenbach, and A. Mudher, "Increased throughput assays of locomotor dysfunction in Drosophila larvae," *J Neurosci Methods*, vol. 203, no. 2, pp. 325–334, 2012.

[12] E. Yemini, R. A. Kerr, and W. R. Schafer, "Illumination for worm tracking and behavioral imaging." *Cold Spring Harb Protoc*, vol. 2011, no. 12, pp. 1480–1482, 2011.

[13] S. Khurana, W.-K. Li, and N. S. Atkinson, "Image Enhancement for Tracking the Translucent Larvae of Drosophila melanogaster," *PloS one*, vol. 5, no. 12, p. e15259, 2010.

[14] X. Jiang, M. Dawood, F. Gigengack, B. Risse, S. Schmid, D. Tenbrinck, and K. Schäfers, "Biomedical Imaging: a Computer Vision Perspective," in *Computer Analysis of Images and Patterns*. Berlin, Heidelberg: Springer Berlin Heidelberg, 2013, pp. 1–19.

[15] A. Gomez-Marin, N. Partoune, G. J. Stephens, and M. Louis, "Automated Tracking of Animal Posture and Movement during Exploration and Sensory Orientation Behaviors." *PloS one*, vol. 7, no. 8, p. e41642, 2012.

[16] G. J. Stephens, B. Johnson-Kerner, W. Bialek, and W. S. Ryu, "Dimensionality and dynamics in the behavior of C. elegans." *PLoS Comput. Biol.*, vol. 4, no. 4, p. e1000028, 2008.

[17] N. A. Swierczek, A. C. Giles, C. H. Rankin, and R. A. Kerr, "High-throughput behavioral analysis in C. elegans." *Nature Methods*, vol. 8, no. 7, pp. 592–598, 2011.

[18] L. Luo, M. Gershow, M. Rosenzweig, K. Kang, C. Fang-Yen, P. A. Garrity, and A. Samuel, "Navigational decision making in Drosophila thermotaxis." *J. Neurosci.*, vol. 30, no. 12, pp. 4261–4272, 2010.

[19] T. Ohyama, T. Jovanic, G. Denisov, T. C. Dang, D. Hoffmann, R. A. Kerr, and M. Zlatic, "High-Throughput Analysis of Stimulus-Evoked Behaviors in Drosophila Larva Reveals Multiple Modality-Specific Escape Strategies," *PloS one*, vol. 8, no. 8, p. e71706, 2013.

[20] A. C. Keene and S. G. Sprecher, "Seeing the light: photobehavior in fruit fly larvae." *Trends Neurosci.*, vol. 35, no. 2, pp. 104–110, 2012.

[21] A. Gomez-Marin, B. J. Duistermars, M. A. Frye, and M. Louis, "Mechanisms of odor-tracking: multiple sensors for enhanced perception and behavior." *Front Cell Neurosci*, vol. 4, p. 6, 2010.

[22] Y. Kawazoe, H. Yawo, and K. D. Kimura, "A simple optogenetic system for behavioral analysis of freely moving small animals." *Neurosci. Res.*, vol. 75, no. 1, pp. 65–68, 2013.

[23] S. Lahiri, K. Shen, M. Klein, A. Tang, E. Kane, M. Gershow, P. Garrity, and A. Samuel, "Two alternating motor programs drive navigation in Drosophila larva." *PloS one*, vol. 6, no. 8, p. e23180, 2011.

[24] A. Kuhara, N. Ohnishi, T. Shimowada, and I. Mori, "Neural coding in a single sensory neuron controlling opposite seeking behaviours in Caenorhabditis elegans." *Nat Commun*, vol. 2, p. 355, 2011.

[25] W. Geng, P. Cosman, C. C. Berry, Z. Feng, and W. R. Schafer, "Automatic Tracking, Feature Extraction and Classification of C. elegans Phenotypes," *IEEE Trans. Biomed. Eng.*, vol. 51, no. 10, pp. 1811–1820, 2004.

[26] D. Ramot, B. E. Johnson, T. L. Berry, L. Carnell, and M. B. Goodman, "The Parallel Worm Tracker: a platform for measuring average speed and drug-induced paralysis in nematodes." *PloS one*, vol. 3, no. 5, p. e2208, 2008.

[27] H. Pistori, V. V. Viana Aguiar Odakura, J. B. Oliveira Monteiro, W. N. Gonçalves, A. R. Roel, J. de Andrade Silva, and B. B. Machado, "Mice and larvae tracking using a particle filter with an auto-adjustable observation model," *Pattern Recognition Letters*, vol. 31, no. 4, pp. 337–346, 2010.

[28] M. Gershow, M. Berck, D. Mathew, L. Luo, E. A. Kane, J. R. Carlson, and A. Samuel, "Controlling airborne cues to study small animal navigation." *Nature Methods*, vol. 9, no. 3, pp. 290–296, 2012.

[29] S. J. Wang and Z.-W. Wang, "Track-A-Worm, An Open-Source System for Quantitative Assessment of C. elegans Locomotory and Bending Behavior," *PloS one*, vol. 8, no. 7, p. e69653, 2013.

[30] X. Heng, D. Erickson, L. R. Baugh, Z. Yaqoob, P. W. Sternberg, D. Psaltis, and C. Yang, "Optofluidic Microscopy - a Method for Implementing a High Resolution Optical Microscope on a Chip," *Lab Chip*, vol. 6, no. 10, pp. 1274–1276, 2006.

[31] S. O. Isikman, W. Bishara, S. Mavandadi, F. W. Yu, S. Feng, R. Lau, and A. Ozcan, "Lens-free optical tomographic microscope with a large imaging volume on a chip." *Proc. Natl. Acad. Sci. U.S.A.*, vol. 108, no. 18, pp. 7296–7301, 2011.

[32] P. Liu, R. J. Martin, and L. Dong, "Micro-electro-fluidic grids for nematodes: a lens-less, image-sensor-less approach for on-chip tracking of nematode locomotion." *Lab Chip*, vol. 13, no. 4, pp. 650–661, 2013.

[33] F. Wang and X. Ren, "Empirical Evaluation for Finger Input Properties In Multi-touch Interaction," in *Proc. 27th Int. Conf. on Human Factors in Comp. Systems*, 2009.

[34] B. Risse, D. Berh, N. Otto, X. Jiang, and C. Klämbt, "Quantifying Subtle Locomotion Phenotypes Of Drosophila Larvae Using Internal Structures Based on FIM Images," *Comp. Bio. and Med.*, 2014 under revision.

[35] A. Yilmaz, O. Javed, and M. Shah, "Object tracking: A survey," *Computing Surveys*, vol. 38, no. 4, 2006.

[36] E. S. Heckscher, S. R. Lockery, and C. Q. Doe, "Characterization of Drosophila larval crawling at the level of organism, segment, and somatic body wall musculature." *J. Neurosci.*, vol. 32, no. 36, pp. 12 460–12 471, 2012.

[37] C. L. Hughes and J. B. Thomas, "A sensory feedback circuit coordinates muscle activity in Drosophila." *Mol. Cell. Neurosci.*, vol. 35, no. 2, pp. 383–396, 2007.

[38] B. Risse, N. Otto, D. Berh, X. Jiang, and C. Klämbt, "FIM imaging and FIMTrack: Two new tools allowing high-throughput and cost effective locomotion analysis," *JoVE*, 2014 under revision.