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5 **Ethoscopes: an open platform for high-throughput *ethomics***

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24 **Authors' contributions**: QG, LGR and GFG designed the platform; QG and LGR wrote the software;

25 QG and EJB performed the experiments; ARJ contributed the optomotor module; ASF contributed the

26 AGO module; all authors contributed the manuscript.

27

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29 **Abstract**

30 We present ethoscopes, machines for high-throughput ethomics in *Drosophila* and other small animals.

31 Ethoscopes provide four unique features: they provide a software and hardware solution that is

32 reproducible, scalable and open-source; they perform not just real-time tracking, but faithful real-time

33 profiling of behaviour using a supervised machine learning algorithm; they can stimulate flies in a

34 feedback-loop mode; they are highly customisable and open source.

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36 Understanding how behaviour is encoded in the brain is one of the ultimate goals of neuroscience. In

37 particular, much of modern neurobiology focuses on finding the genes and the neuronal circuits

38 underlying simple and complex behaviours alike, aiming to describe and ultimately understand how the

39 brain processes sensory inputs into motor outputs. For many years, starting from Seymour Benzer's

40 seminal work[1] , the fruit fly *Drosophila melanogaster* has been considered the model organism of

41 choice to dissect the genetics of behaviour. In the past decade, *Drosophila* has also emerged as an

42 excellent model for studying not only the genes, but the neuronal circuitry of behaviour too: the

43 combination of a rapidly delineating connectome together with an unrivalled repertoire of genetics

44 tools has established fruit flies as the most promising animal model to study neuronal circuits.

45 Optogenetics, thermogenetics, a genome wide collection of RNAi lines, and a plethora of crafted and

46 carefully described GAL4 lines constitute an unprecedented arsenal for neurobiologists interested in

47 studying the neuronal circuitry underpinning behaviour. The limiting factor for *ethomics* – the high-

48 throughput approach to behavioural studies - is therefore not the availability of genetic tools, but the

49 access to an objective, reproducible and scalable system to detect and categorise behaviour. In the past

50 few years, several computational approaches were introduced to address this limitation: some

51 specifically dedicated to a subset of behaviours, like sleep[2–4], and others designed to be more versatile[5–

52 8]. However, while computer-assisted analysis of behaviour has the potential of revolutionising the field,

53 adoption and throughput of currently available techniques are limited by several factors: among others,

54 the scope and versatility of the software itself and the requirement for a non standardised hardware-

55 setup, with relevant problems of cost, footprint, and scalability. Here we present a tool aimed at solving

56 these issues, providing an affordable and versatile complete suite to study *ethomics*: ethoscopes.

57   An ethoscope is a self-contained machine able to either record or detect in real-time the activity

58 of fruit flies (and potentially other animals) using computerised video-tracking. It relies on an

59 independent small single-board computer (Raspberry Pi[9]) and a high-definition camera (Raspberry Pi

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60   camera) to capture and process infrared-illuminated video up to a resolution of 1920x1080, at 30

61   frames per second (**Fig. 1a**). Ethoscopes are assembled in a 3D-printed case measuring approximately

62   10x13x19 cm (**Fig. 1b**). Although we recommend 3D printed assembly for research-grade use, we also

63   provide detailed instruction to build a fully functional ethoscope out of LEGO bricks (**Fig. 1c**, -

64   LEGOscope **Supplementary Material S1**) or out of folded cardboard (**Fig. 1d**, - PAPERscope

65   **Supplementary Material S2**). These latter two options are mainly aimed at education and outreach but

66   they may also be adopted in a real laboratory environment. The technical drawings required to 3D-print

67   and assemble an ethoscope, along with its software (Python code on a Linux instance) are released

68   under the open source GPL3 license and freely available on the ethoscope website

69   (https://lab.gilest.ro/ethoscope). The combination of consumer-grade electronics, 3D printing and free

70   open source software results into a total cost of about £80 for each machine. Limited cost, along with

71   the fact that each ethoscope relies on its own computing power, allows for easy scaling of the entire

72   platform.

73   In a typical usage scenario, several ethoscopes are placed in a climatic controlled chamber, each

74   powered through a USB cable; ethoscopes connect via WIFI to a computer acting as data collecting

75   station ("the hub" in **Supplementary Fig. 1a**) and are controlled remotely via a graphical web interface

76   (**Supplementary Fig. 1** and **Supplementary Video S1**). If the hub is connected to the internet, the

77   entire platform will receive automatic software updates from a central GIT repository. Flies to be

78   tracked are loaded into a behavioural arena that slides and locks inside the lower part of the ethoscope

79   (**Fig. 1a**). Alike the rest of the machine, arenas are also 3D-printed and the design of the arena to be

80   employed depends on the nature of the experiment: some examples of arenas developed in our

81   laboratory are provided in **Fig. 1e-k** and span arenas adopted for long term sleep experiments that may

82   be lasting weeks (**Fig. 1e-g,j**) or short term assays such as decision making (**Fig. 1h**) and courtship

83   (**Fig. 1i,k**). When starting an experiment, the experimenter can decide whether the activity of the

84   animals should be tracked in real-time or whether the ethoscope should record a video to be analysed at

85   a later time, with the ethoscope software or with another software of choice. In real-time tracking

86   mode, ethoscopes will detect and record the position and orientation of each animal with a variable

87   frame rate of 1-4 frames per second (**Fig. 2a-b**).

88   To validate the accuracy of tracking, we recorded 2736 hours of video and asked three

89   independent experienced fly researchers to manually annotate the position of flies in 1413 frames. We

90   then compared the manually annotated positions to the positions detected by ethoscopes, and found that

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91 the median distance between the two was 0.3 mm, corresponding to a tenth of a fly body length. In no

92 cases, did the error exceed one body length (2.5mm). To enrich the capabilities of ethoscopes, we also

93 implemented a real-time behavioural annotator. We created a ground-truth of 1297 videos, each lasting

94 10 seconds and each manually annotated by at least three experienced fly researchers (**Fig. 2c**,

95 annotation labels were: "walking", "micro-movement" or "immobile"). Random forest variable

96 importance[10] was used to screen for possible predictors of mobility in a supervised manner and the two

97 highest-ranking features - maximal velocity and cumulative walked distance – were selected for further

98 analysis (**Fig. 2d,e**). Conveniently, ROC analysis showed that maximal velocity alone appeared to

99 serve as faithful predictor of behaviour (**Fig. 2d,e**). Therefore, not only can ethoscopes annotate the

100 position of flies, but also detect in real-time when an animal is immobile, performing a micro-

101 movement (such as grooming or eating), or walking, with an accuracy of 94.3% for micro-movement

102 detection and 99.0% for walking detection (accuracy = (N_true_positive + N_true_negative) / N).

103   The ability to recognize simple behaviour in real-time opens a new perspective: animal

104 -dependent feedback-loop stimuli delivered upon behavioural trigger. Interfering with behaviour

105 through external stimuli is an important tool for neuroscientists as it pushes beyond description and

106 allows for more manipulative analysis. In principle, feedback loops can be used for multiple purposes:

107 to reinforce learning, to sleep deprive animals, to stimulate or silence circuits using optogenetics, to

108 study operant conditioning, *etc*. Systems operating feedback-loop stimuli on fruit flies were proposed

109 before and already proved to be useful, but they are not easily compatible with a high-throughput

110 approach and are focused on very specific usage[11,12]. We therefore designed ethoscopes so that they

111 could be extended with modules that seamlessly connect with the machine and react to real-time

112 analysis to trigger an action whenever a condition is satisfied. Figure 3 shows three examples of such

113 modules: an air/gas/odour (AGO) delivery module (**Fig. 3a,b**), a rotational module capable of variable

114 intensity(**Fig. 3d,e**), and an opto-motor module combining optogenetic stimulation and motor

115 disturbance (**Fig. 3g,h**). Modules plug into the bottom part of the machine and are configured through

116 the graphical web-interface, where the experimenter can set the trigger conditions that will activate the

117 stimulus. A trigger can be a combinatorial ensemble of position, time, and behaviour (e.g. "*micro-*

118 *movement for at least 20 seconds within 5mm from the food*" or "*immobile for at least 5 minutes*

119 *anywhere*"). As proof of principle, we provide representative evidence of how single flies react to three

120 different stimuli: a 5 seconds delivery of CO_2, triggered by crossing of the midline tube (**Fig. 3c**); a 2

121 seconds fast rotation of the tube, triggered by 20 seconds of immobility (**Fig. 3f**); a 5 seconds opto-

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122 stimulation on "moon-walker"[13] receptive flies, manually triggered (**Supplementary Video S2**). In
123 general, flies responses to stimuli will obviously depend on the experimental paradigm of choice. On
124 the ethoscope website, we provide detailed instruction on how to build all three modules and a
125 description of the API needed to interface any new custom module to the ethoscope platform.
126 Ultimately, we expect and encourage users to build modules based on their own scientific needs. One
127 unprecedented strength of the feedback-loop module system is the ability to interact with single flies
128 rather than with the entire population, which provides, among other things, the option of performing
129 sham treatments.

130   Ethoscopes emerge from the maker culture to combine three of the most revolutionary
131 innovations of the last decades - 3D printing, small single-board computers and machine learning - into
132 a novel paradigm for behavioural researchers. They were designed to be easy to build, inexpensive,
133 compatible with high-throughput research, and able to generate reproducible results. Moreover, in their
134 LEGO and paper versions, they can serve as excellent tool for education and citizens science.
135 Ethoscopes rely heavily on Raspberry Pis, the third best-selling computer of all time, currently running
136 at their 3rd hardware version. We expect Raspberry Pis to continue in their evolution, and we therefore
137 expect ethoscopes' computing power to grow accordingly. A standardised, plug-and-play, inexpensive
138 tool for behavioural analysis – like ethoscopes are – can be instrumental for future development of the
139 behavioural field, similarly to how activity monitors have been instrumental for the success of
140 circadian biology.

141

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154   **Figure Legends**

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156   **Figure 1 |** The ethoscope. (**a**) Exploded drawing of a prototypical ethoscope. The machine is composed

157   of two main parts: an upper case containing the Raspberry Pi and its camera, and a lower case

158   providing infrared light (IR) and support for the experimental arena. The two cases are separated by

159   spacers maintaining a fix focal distance (140mm). (**b**) A rendered drawing of the assembled model

160   showing actual size. The arena slides in place through sliding guides and locks into position. A webGL

161   interactive 3D model is available as **Supplementary Material 1**. (**c**) The LEGOscope, a version of the

162   ethoscope entirely built using LEGO bricks. A detailed instruction manual is provided in

163   **Supplementary Material 2**. (**d**) A paper and cardboard version of the ethoscope, assembled using

164   220gsm paper and 1mm grayboard. Blueprints are provided in **Supplementary Material 3**. In all

165   cases, ethoscopes must be powered with a 5Vdc input using a common USB micro cable either

166   connected to the main or to a portable power-pack. Cables not shown for sake of simplicity. (**e-k**)

167   Versatility of use with custom behavioural arenas. Examples of 7 different behavioural arenas

168   developed in our laboratory. (**e**) Sleep arena. Most commonly used arena for sleep studies, lodging 20

169   individual tubes. (**f**) Long tubes arena. Used for odour delivery studies or, more generally, for

170   behaviours where longer walking is required. (**g**) Food bullet arena. Animals are placed directly on the

171   arena and food can be replaced by pushing in a new bullet. (**h**) Decision making arena. Can be used to

172   study simple decision making behaviours. (**i**) Square wells arena. Can be used for courtship assay or to

173   record activity in a bi-dimensional environment. (**j,k**) Conceptually analogs to E and I, but designed to

174   work in high-resolution (full-HD) settings. Note that all arenas are marked with three visible reference

175   points (indicated by a red circle in **e.**) that are used by the ethoscope to automatically align images for

176   tracking, providing a degree of physical flexibility.

177

178   **Figure 2 |** Tracking and validation of behavioural classification. (**a**) In real-time tracking mode,

179   ethoscopes record Cartesian coordinates (x,y) of each animal relative to their ROI (region of interest),

180   along with the numbers describing an ellipsis circumscribing the animal (w,h,φ). (**b**) A screenshot of

181   the data table recorded by ethoscope, showing four data points for a single fly. (**c**) To build a statistical

182   model of activity, we used ethoscopes to record offline 2736 hours of video (144 hours x 19 flies) at

183   resolution of 1280x960pixels and frame rate of 25FPS. Video fragments of the duration of 10 seconds

184   were sampled every hour for all 19 animals and then scored by at least three experienced fly

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185   researchers in a randomized order. Consensual annotations – with a margin greater than 0.5 – were

186   kept, resulting in a ground truth of 1297 video fragments (116 ambiguous annotations were excluded

187   using this latter criteria). Scorers manually annotated both the position of the animal in the tube and the

188   perceived behavioural state (i.e. immobile, micro-moving or walking). Ethoscope video-tracking were

189   run independently on the whole video resampled at 1-5FPS, all realistic frame rates for real-time

190   analysis. (**d**) Performance of maximal velocity and cumulative walked distance as features for 1297

191   annotated videos of behaviours. (**e**) Marginal distribution of maximal velocity for each behaviour,

192   showing the thresholds used to detect movement (dotted line) and walking (dashed line). (**f**) ROC

193   curves for movement detection. A conservative threshold of 0.36mm/s, indicated by a red dot on the

194   curve, yields a sensitivity (TPR) of 91.26% and specificity (1-FPR) of 99.65%. (**g**) ROC curve for

195   walking detection. A threshold of 0.9mm/s (red dot on the curve) results in sensitivity and specificity of

196   99.34 and 99.39, respectively.

197

198   **Figure 3 |** Versatility of use with behavioural feedback-loop modules. (**a**) Diagram and (**b**) detail of the

199   air/gas/odour (AGO)-delivery module. Two independent air flows (blue and purple in the drawing) are

200   fed into the module using external sources. The module features 10 LEGO valves, each independently

201   controlled through a servo motor. The motor switches the air source on the valve, selecting which

202   source will be relayed to the tube containing the fly. Available positions are: blue source, purple source,

203   no source. (**c**) Representative response of three flies subjected to CO_2 administration using the AGO

204   module. CO_2 release lasts 5 seconds (gray bar) and it is triggered by midline crossing (red dot). The

205   blue line indicates the fly position in the tube over the 150 second period. (**d**) Model and (**e**) detail of

206   the rotational module. The module employs a servo motor to turn the tube hosting the fly. Direction,

207   speed, duration and angle of the rotation can be modulated to change the quality of the stimulus. (**f**)

208   Representative response of three flies upon stimulation using the rotational module shown in (**d,e**).

209   Rotation of the tube is triggered by 20 consecutive seconds of immobility (dashed line) and it is

210   followed by 5 seconds of masking during which tracking is suspended to avoid motion artefacts (cyan

211   area). The bottom panel shows traces of a dead fly. (**g**) Model of the optomotor module, able to

212   simultaneously stimulate single flies with motion and light. (**h**) Detailed view of the optomotor

213   principle. Light is directed into the tube using optical fiber. **Supplementary Videos 2** shows the

214   optomotor module in action.

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216 **Supplementary Figure S1 |** The ethoscope platform. (**a**) A diagram of the typical setup. Ethoscopes,

217 powered through a USB adapter, are connected in an intranet mesh through an Access Point (AP) or a

218 WI-FI router. A server computer in the network acts as hub, downloading data from ethoscopes and

219 serving a web-based user interface (UI). Ethoscopes can be controlled through the web-UI, either

220 locally or remotely. (**b**) Screenshot of the homepage of the web-UI, showing a list of running machines

221 and some associated experimental metadata (e.g. username and location). (**c**) Screenshot of an

222 ethoscope control page on the web-UI, providing metadata about the experiment and a real-time

223 updated snapshot from the ethoscope point of view.

224

225 **Supplementary material 1 |** Interactive 3D rendering of the assembled ethoscope – requires webGL

226 capable browser (e.g. Google Chrome)

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228 **Supplementary material 2 |** Instruction booklet for building a LEGOscope

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230 **Supplementary material 3 |** Instruction booklet for building a PAPERscope

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232 **Supplementary Videos S1 |** An overview of how the ethoscope platform works

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234 **Supplementary Videos S2 |** The optogenetics component of the optomotor module in action.

235 Moonwalking flies (VT200107-Gal4 :: UAS-CsChrimson) are illuminated for 5-7 seconds using a red
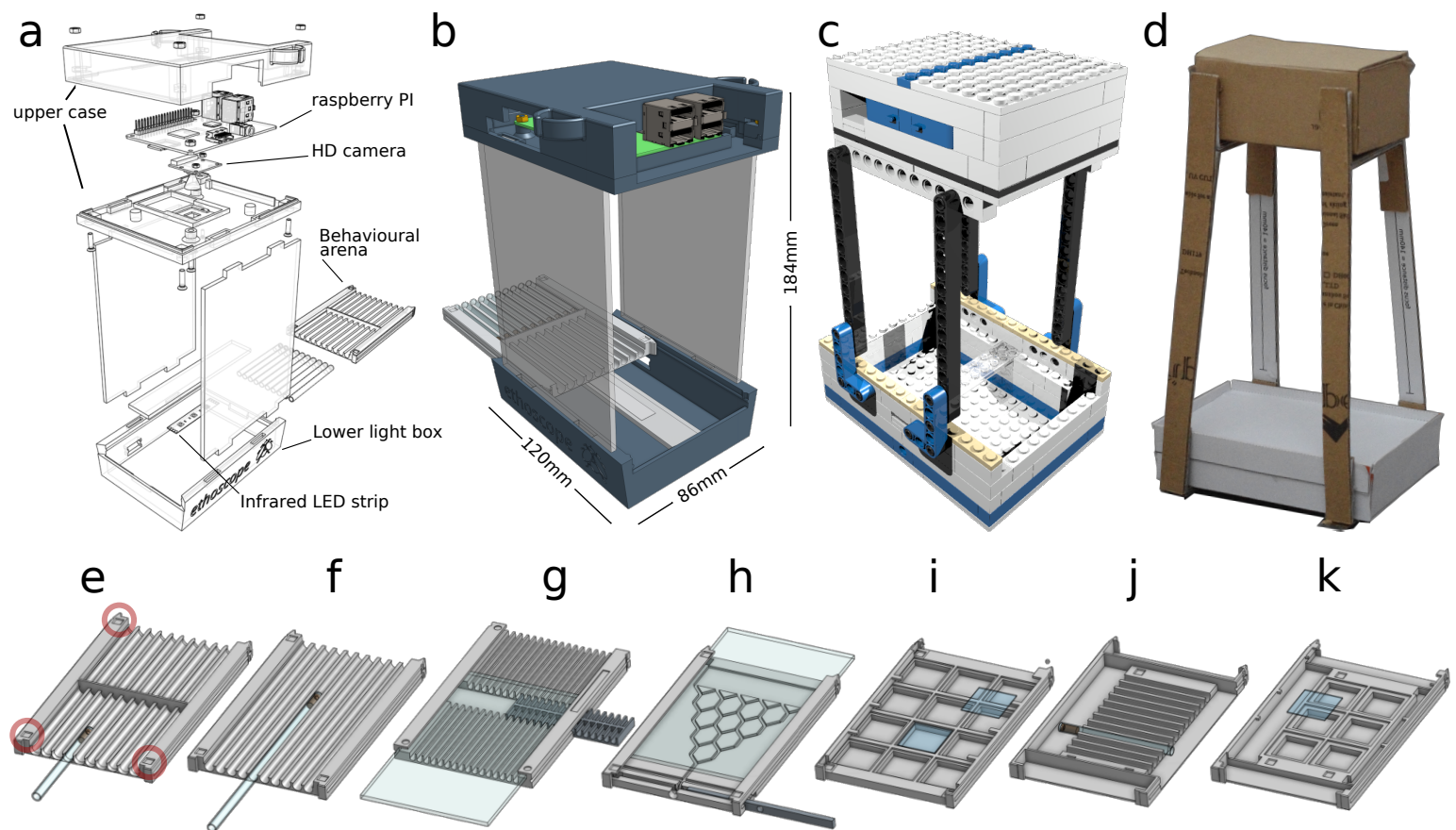
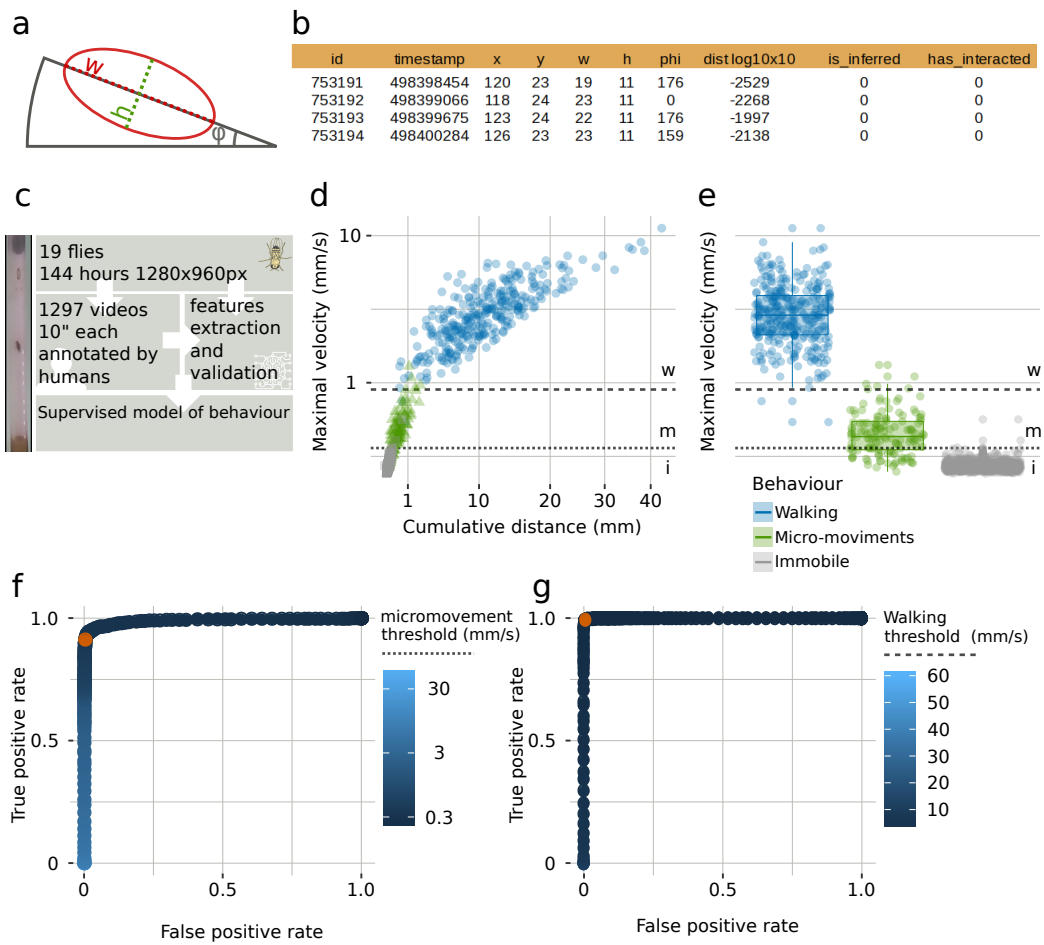236 LED (630nm) through an optical fibre.

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a

upper case

raspberry PI

HD camera

Behavioural arena

Lower light box

Infrared LED strip

b

184mm

120mm

86mm

c

d

e f g h i j k

Figure 1

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Figure 2

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a

d

g

b

servo motor

LEGO valve

e

rubber o-ring

servo motor

*180º rotation
variable speed*

h

geared DC motor

100-400rpm

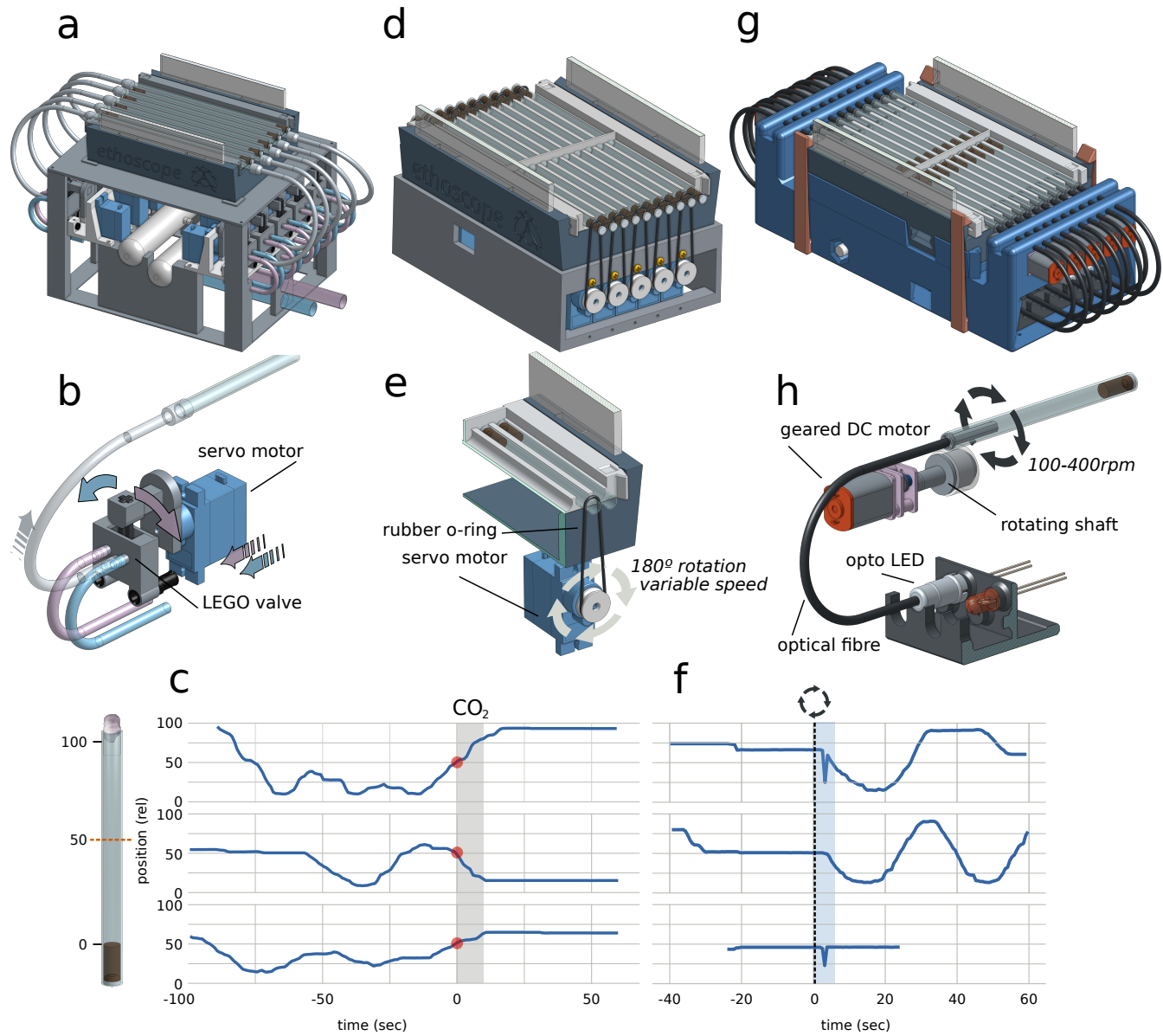rotating shaft

opto LED

optical fibre

c

position (rel)

CO_2

time (sec)

f

time (sec)

Figure 3

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