

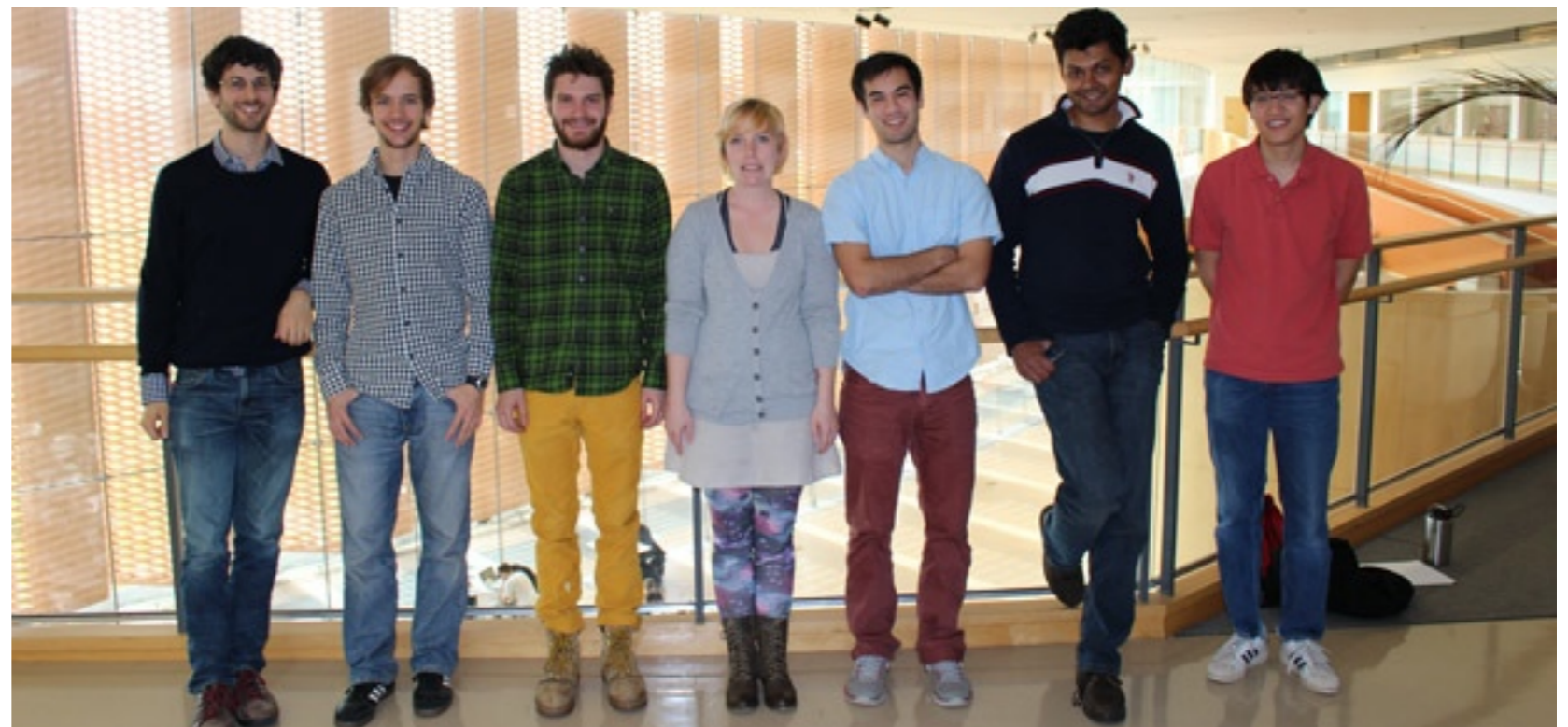
# Optical neurophysiology in freely moving *C. elegans*

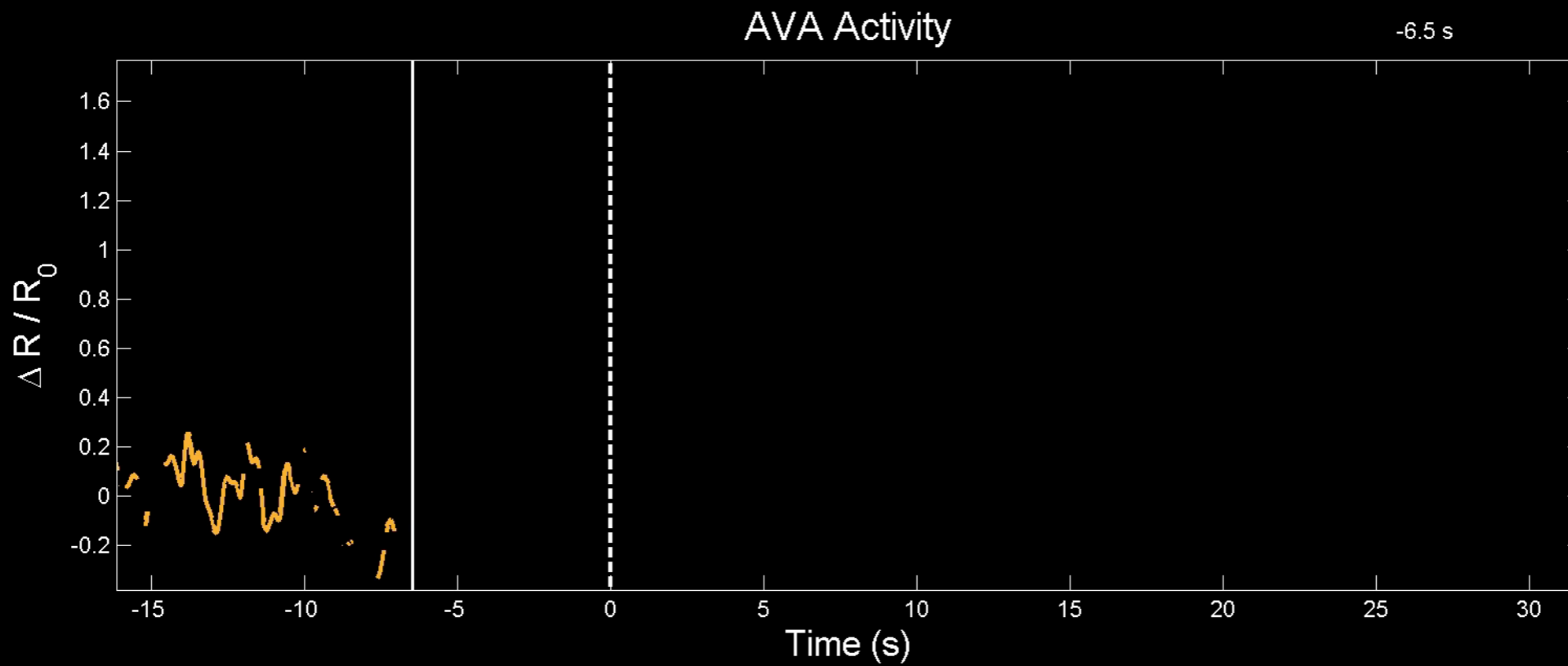
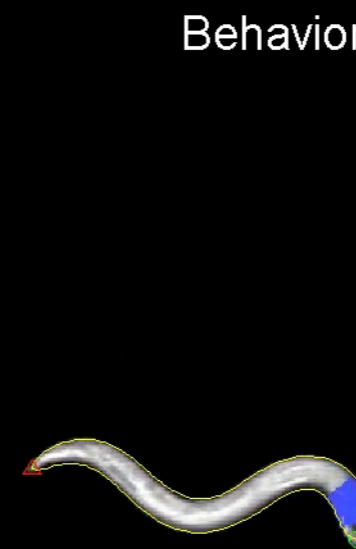
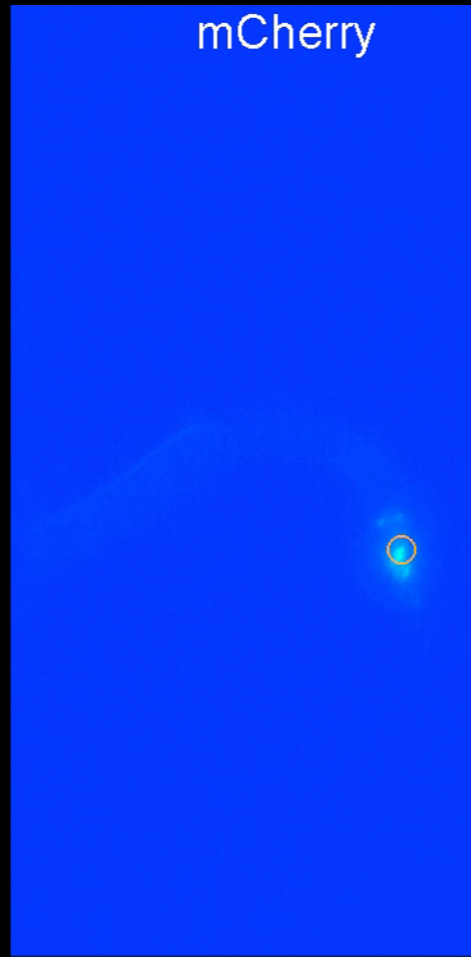
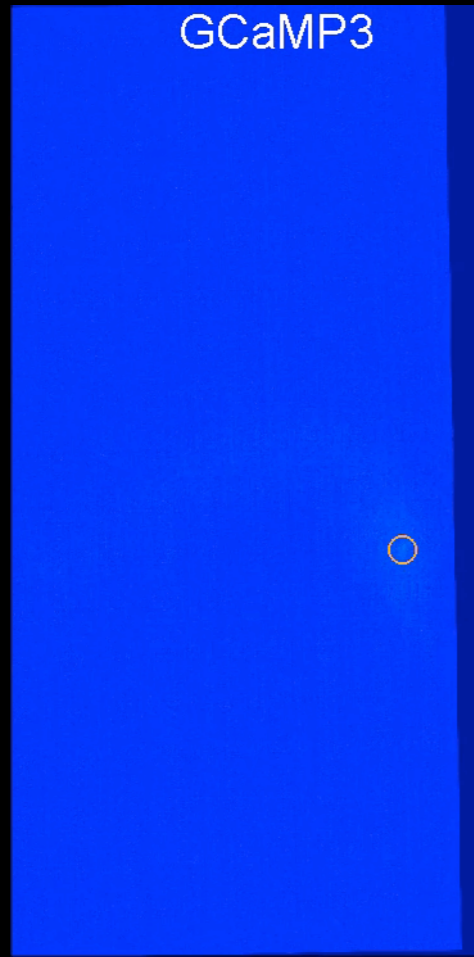
**Andrew Leifer**

Lewis-Sigler Institute

<http://leiferlab.princeton.edu>

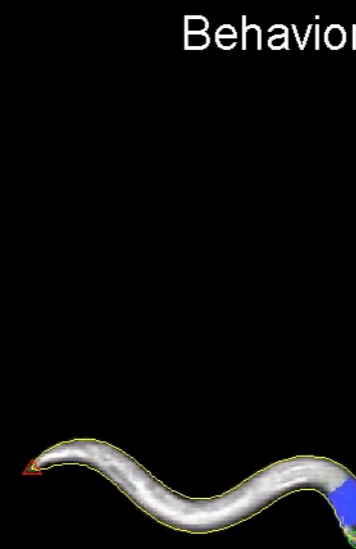
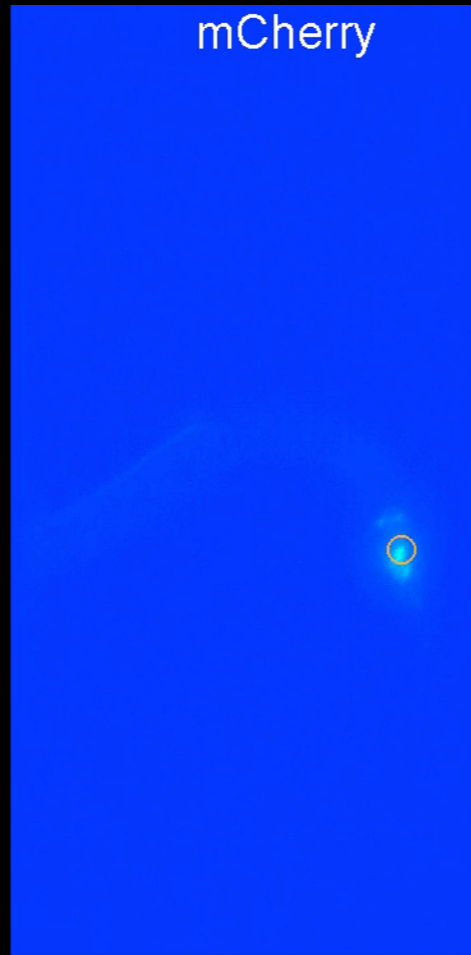
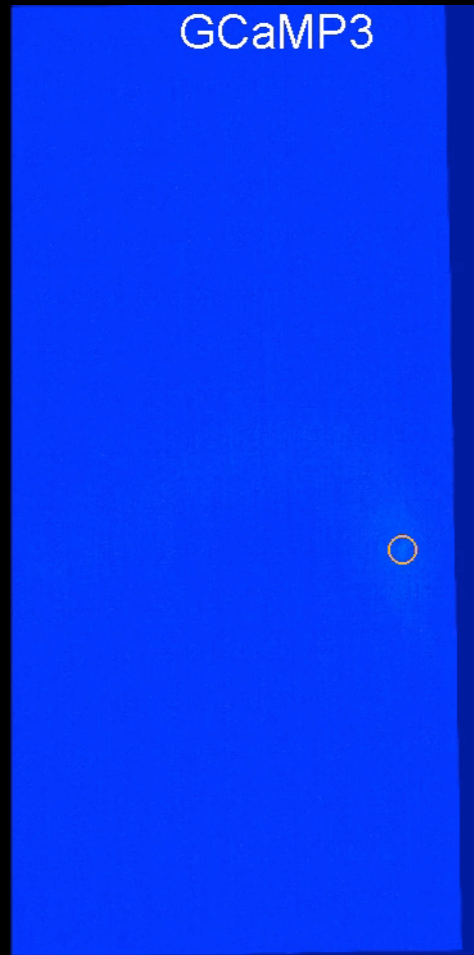
*Ce Neuro Conference*  
*University of Wisconsin, Madison*  
*9 July 2014*





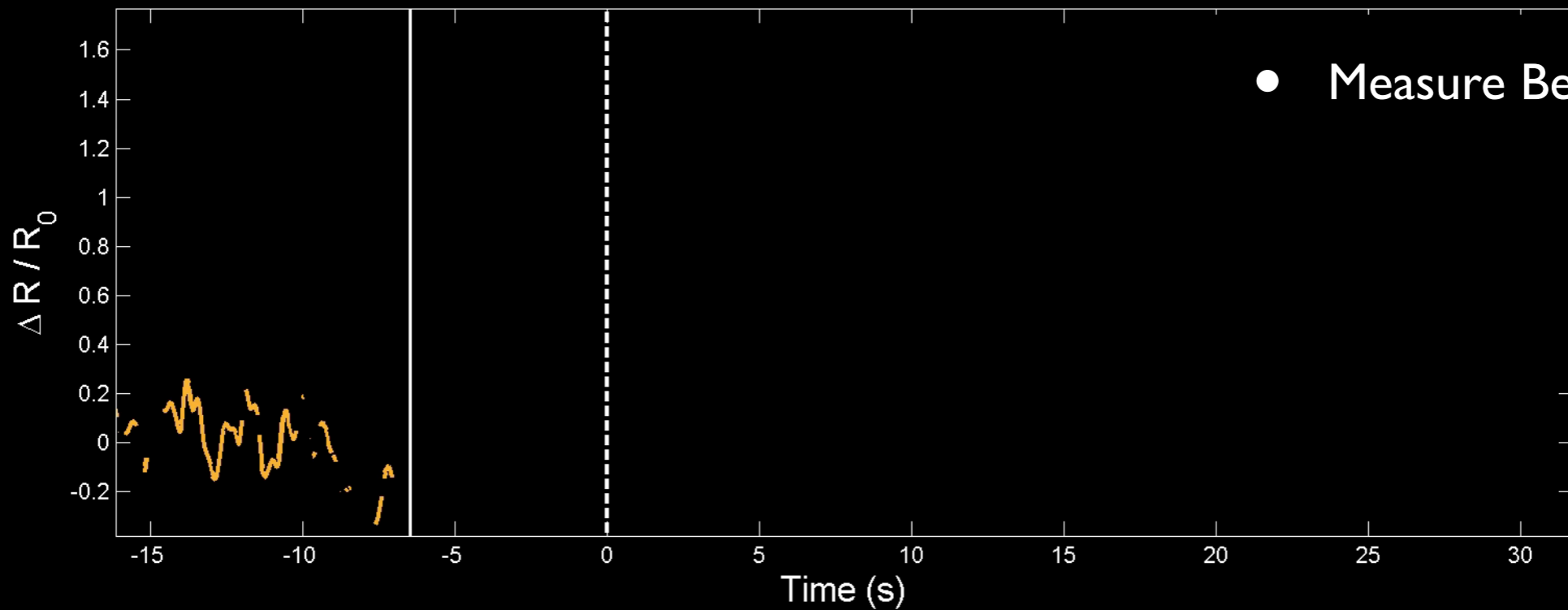
*mec-4::ChR2, rig-3::GCaMP3::sl2::mCherry*

Shiple et al., *Front Neural Circuits*, 2014



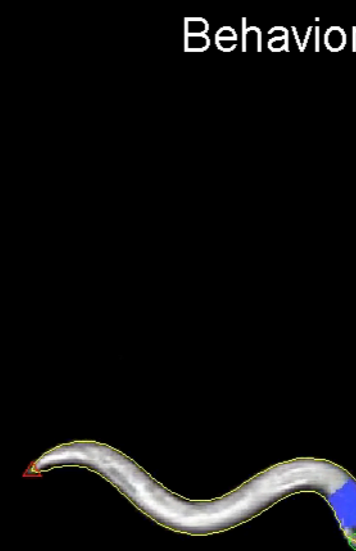
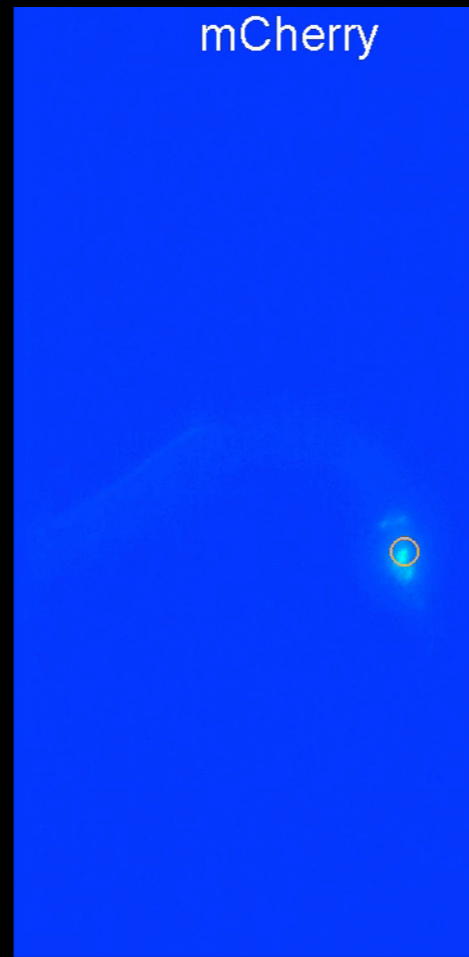
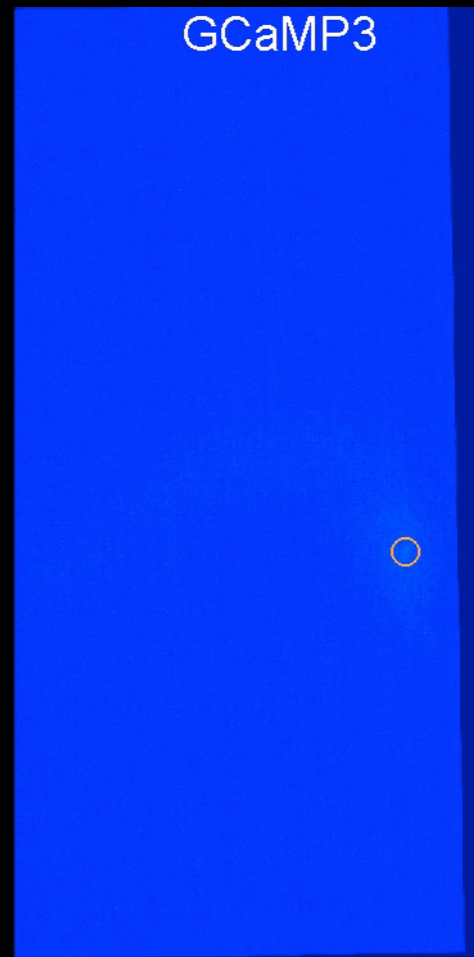
AVA Activity

-6.5 s



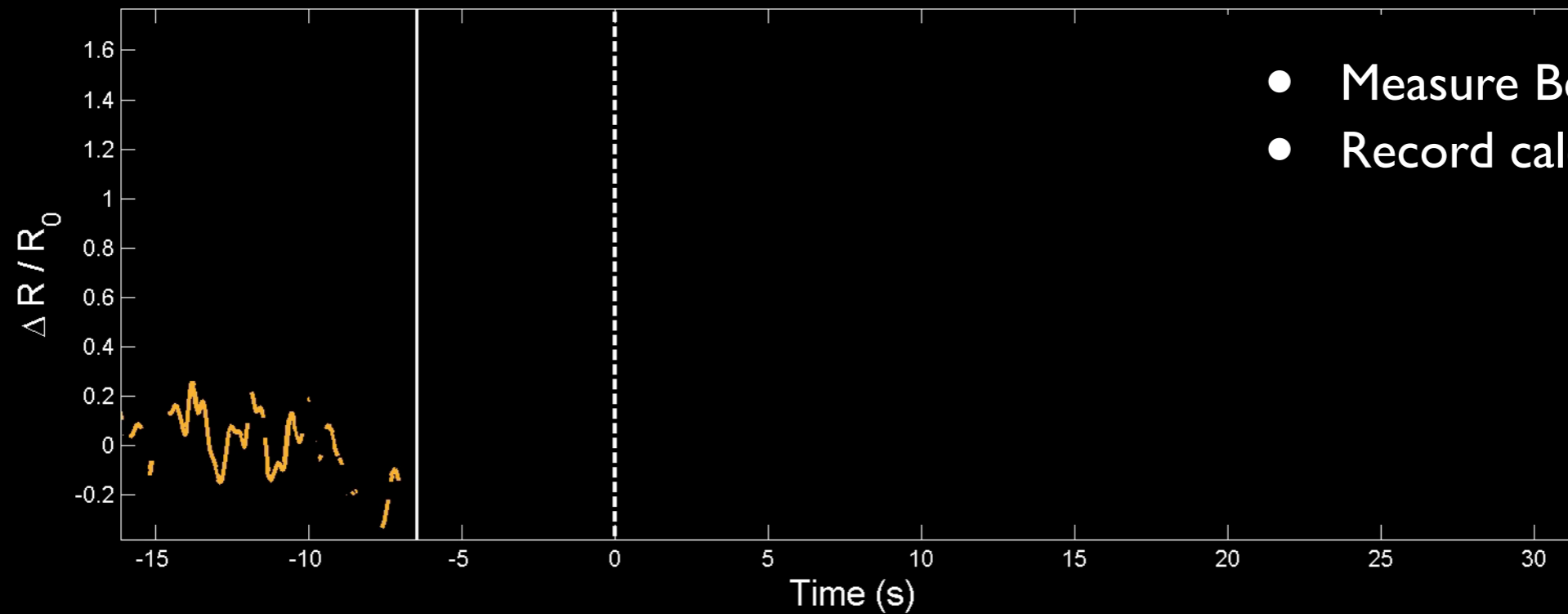
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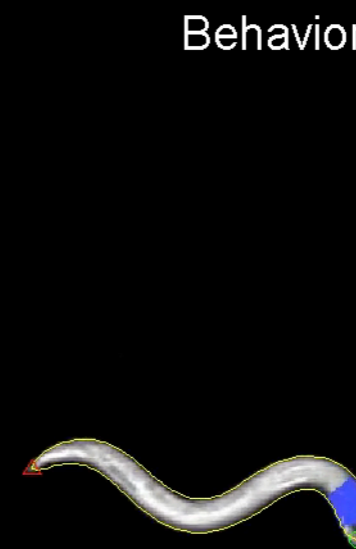
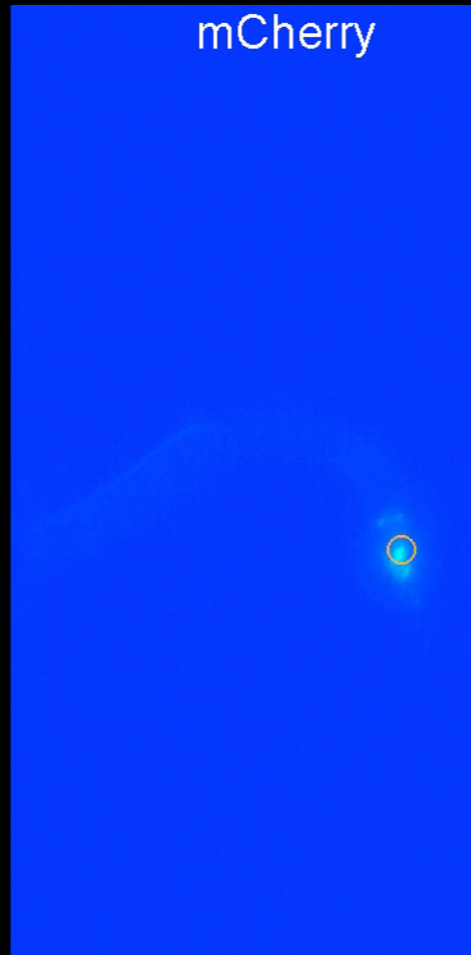
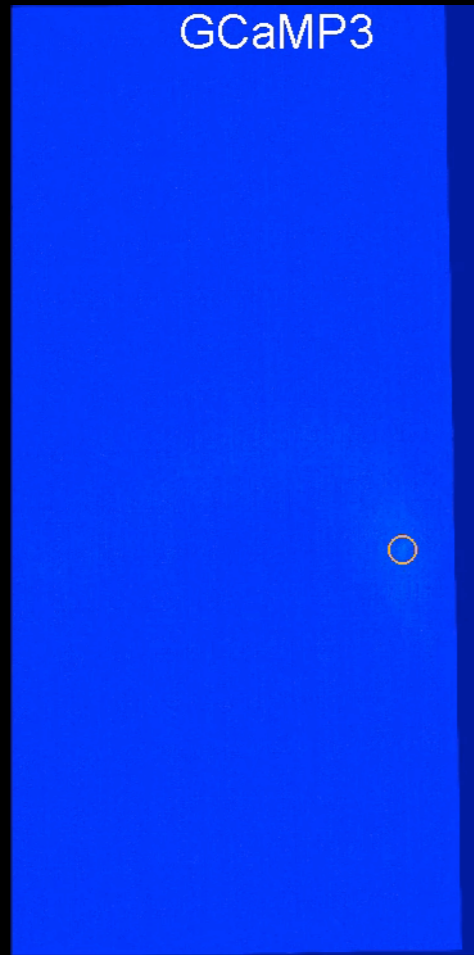
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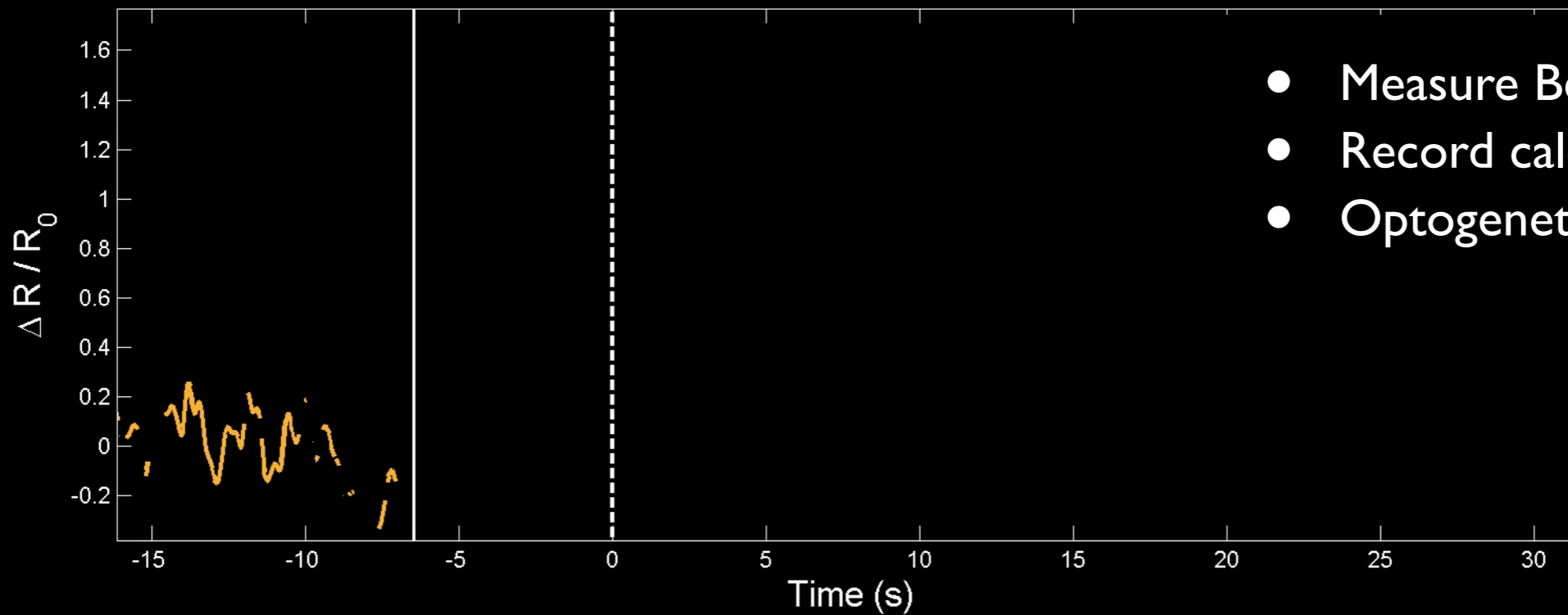
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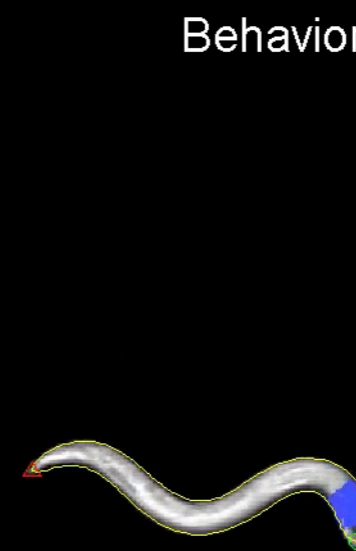
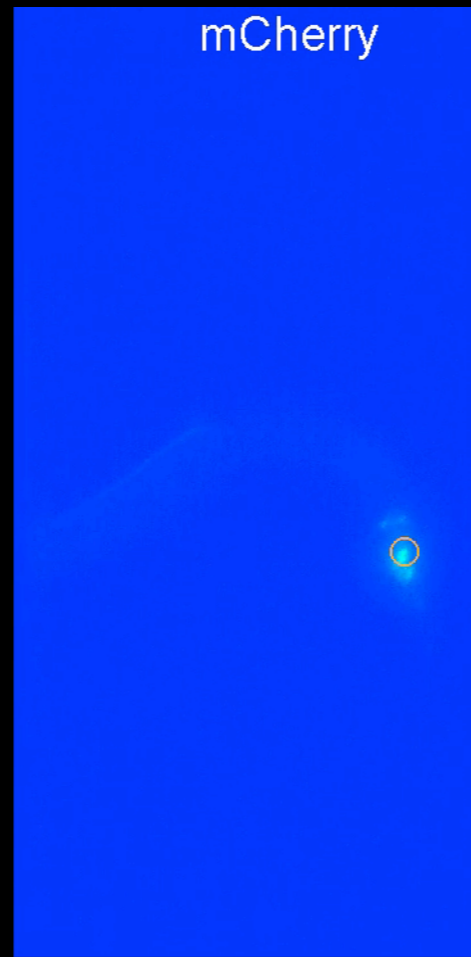
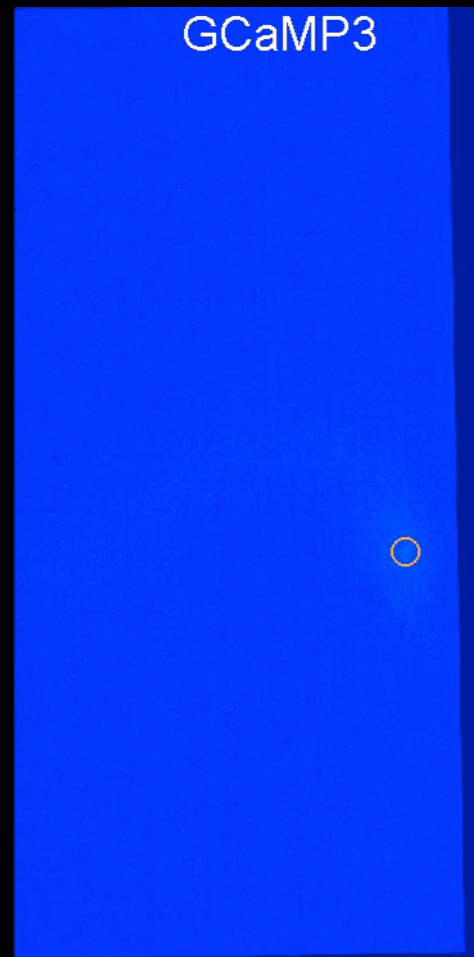
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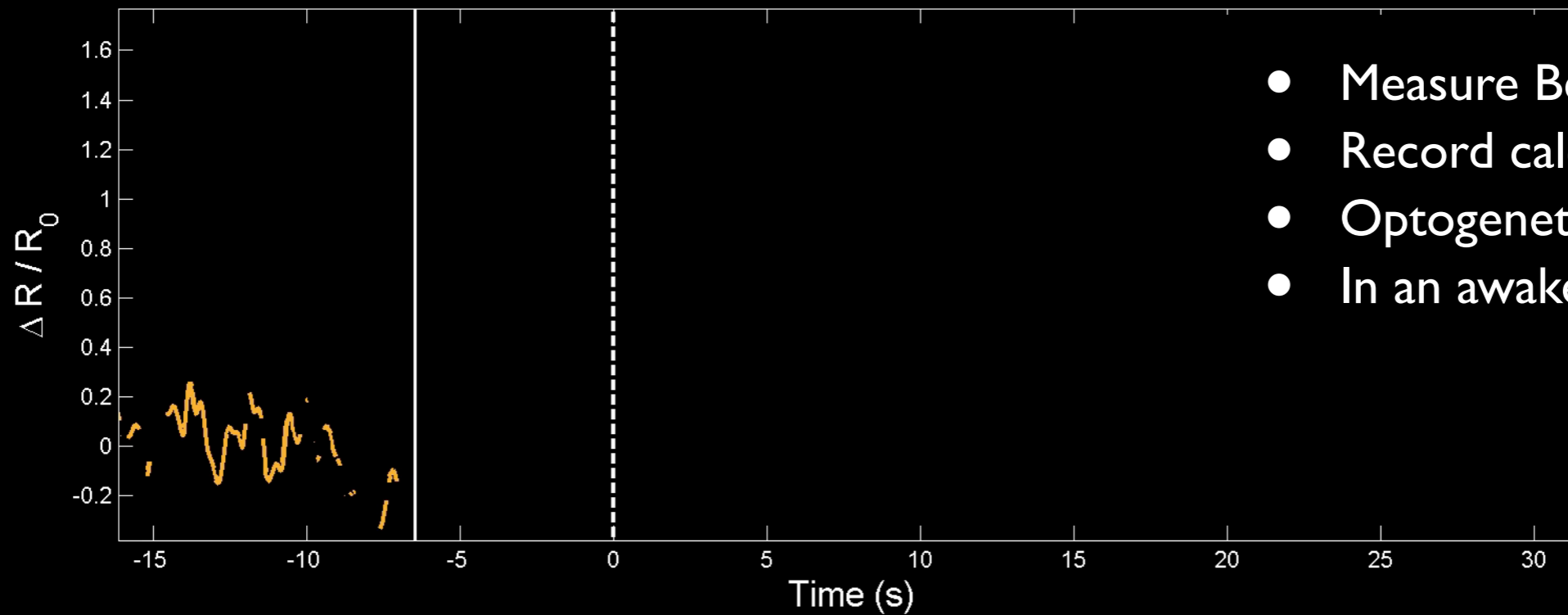
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AVA Activity

-6.5 s



- Measure Behavior
- Record calcium activity
- Optogenetically stimulate
- In an awake behaving animal

*mec-4::ChR2, rig-3::GCaMP3::sl2::mCherry*

Shiple et al., *Front Neural Circuits*, 2014

# Why study neural activity in free moving worms?

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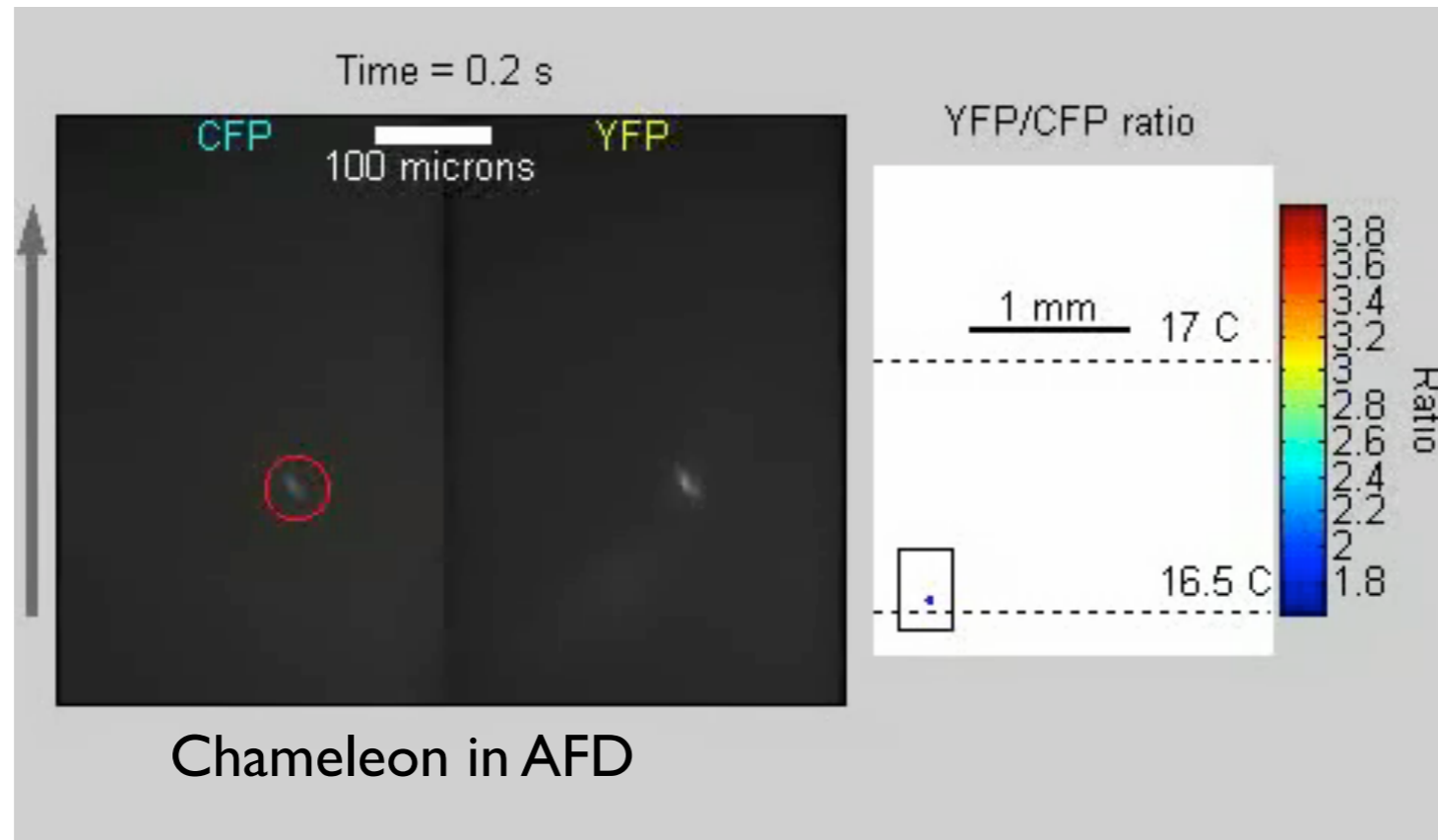
- Working in a behaving animal is the only way to directly probe neural coding of behavior
- Genetic or laser ablation studies lack insights into neural *dynamics*
- .. and the tools are finally available

# Outline

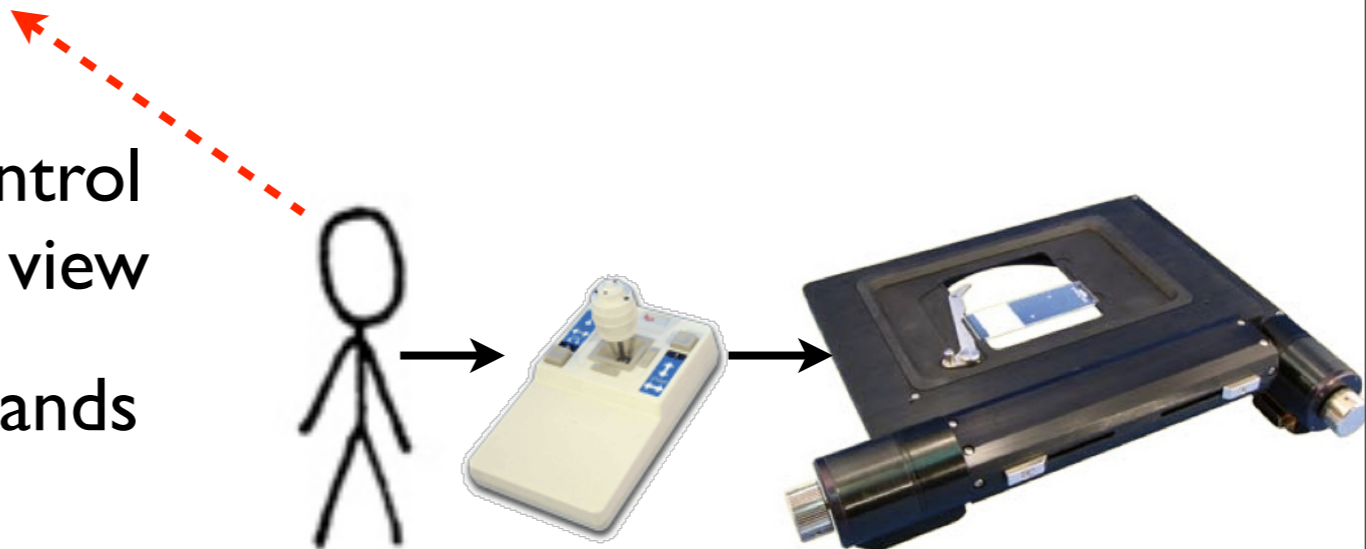
- Give a broad overview of existing methods in freely moving worms for
  - Optogenetics
  - Calcium imaging
- Discuss practical matters for adopting these techniques in your lab
- Thoughts about the future

Note: no discussion of scientific results

# Optical neurophysiology in moving worms requires real-time tracking



- At a minimum feedback is needed to control a stage to keep the worm in the field of view
- A human can provide feedback: steady hands and patience



Clark et al, *J. of Neuroscience* 2007.

# Computer vision software can track the worm and control stage position

Real-time computer vision software based on worm outline.

First implemented by Ben Arous et al., 2010; (Schafer lab)

Same strategy can be used for tracking or for generating targeted illumination patterns

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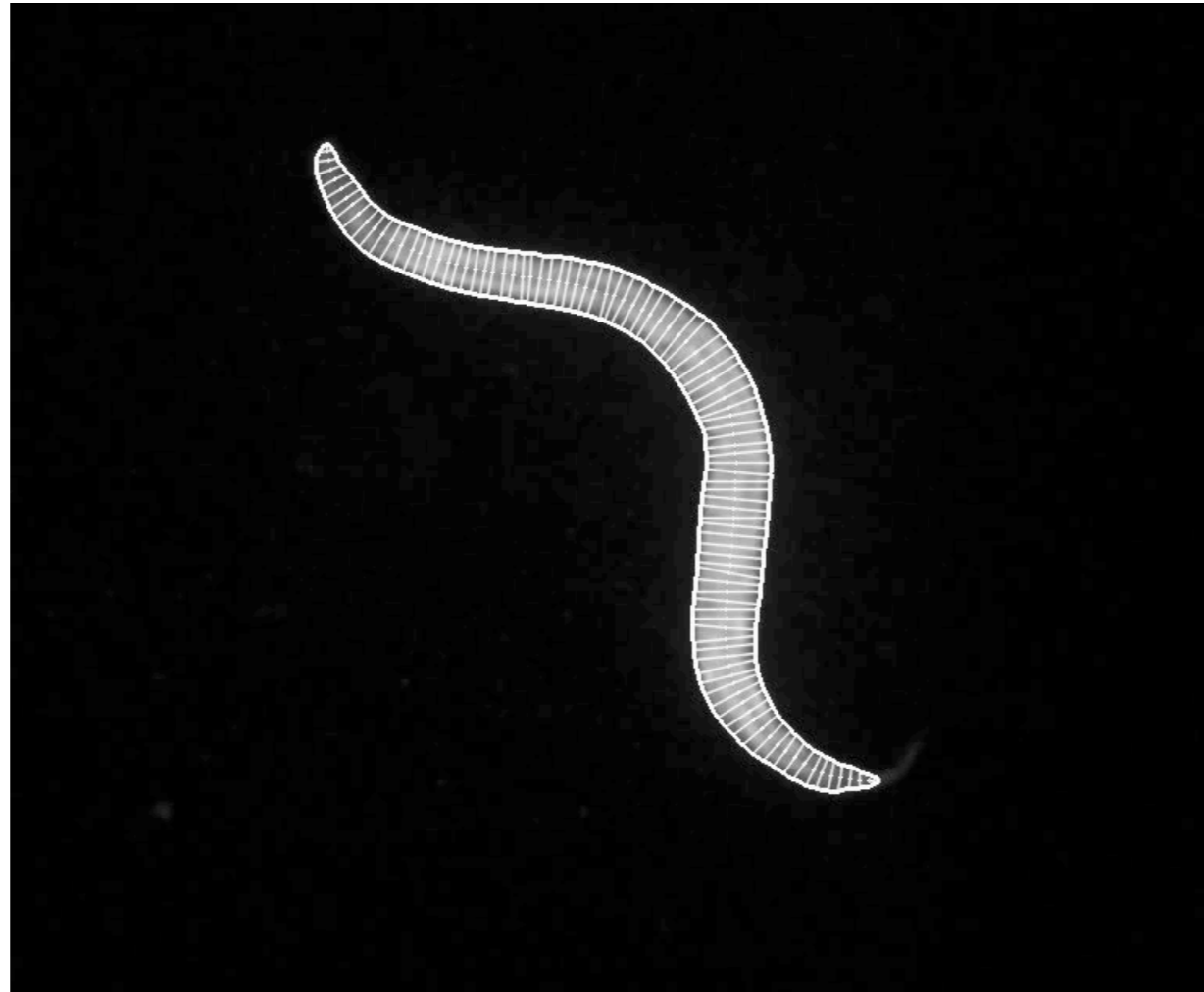


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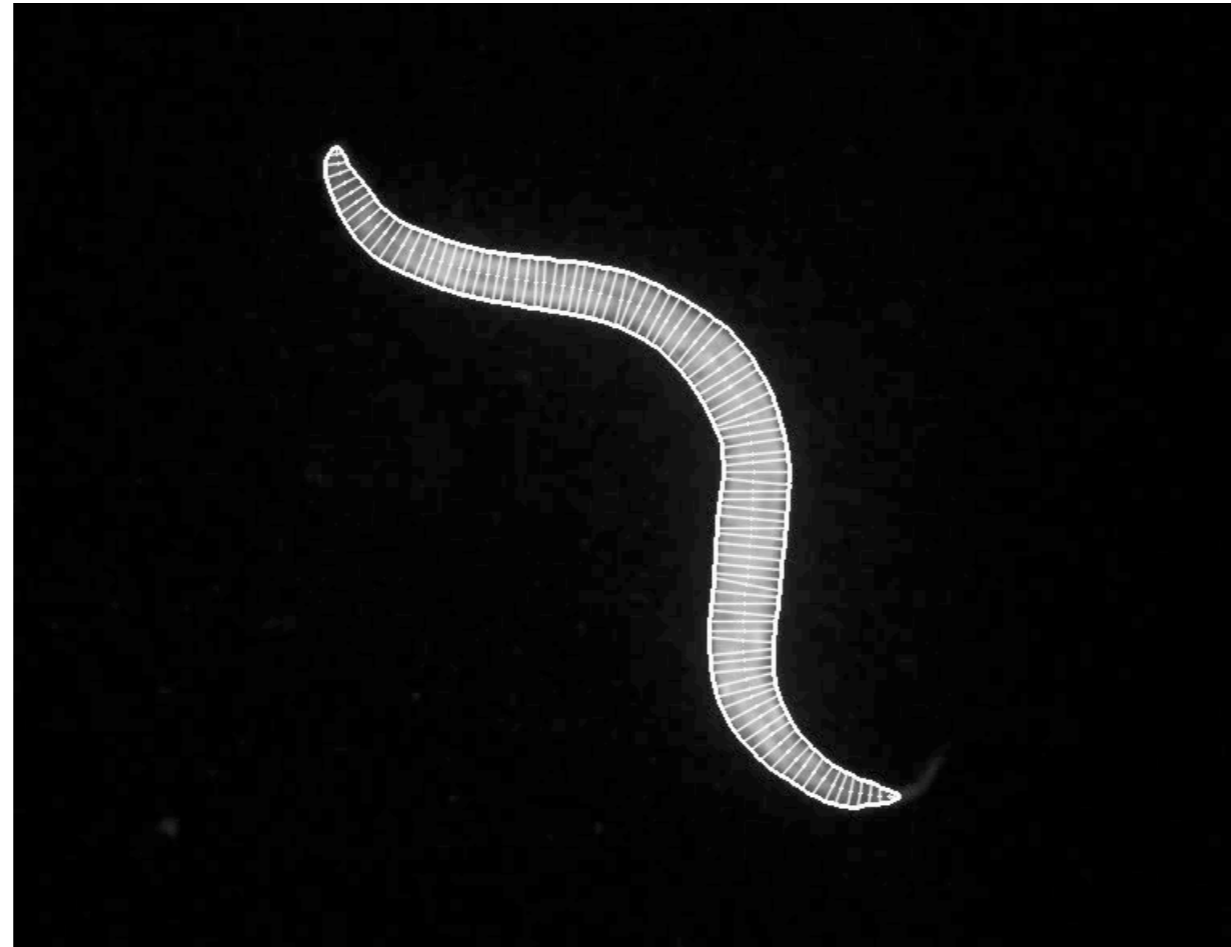
Code:  
<http://git.io/colbert>

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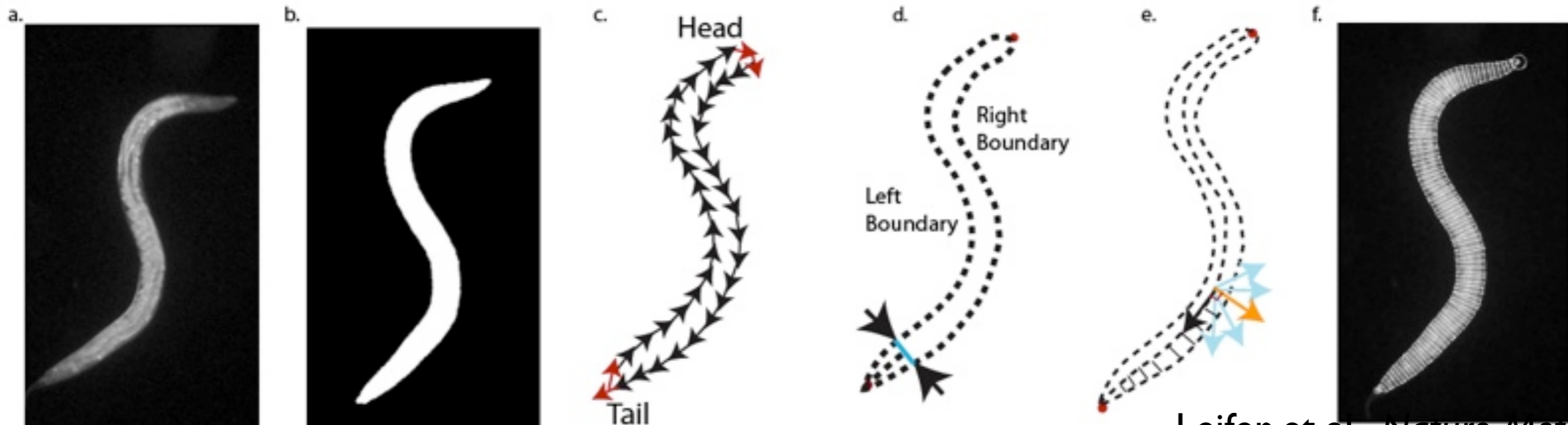
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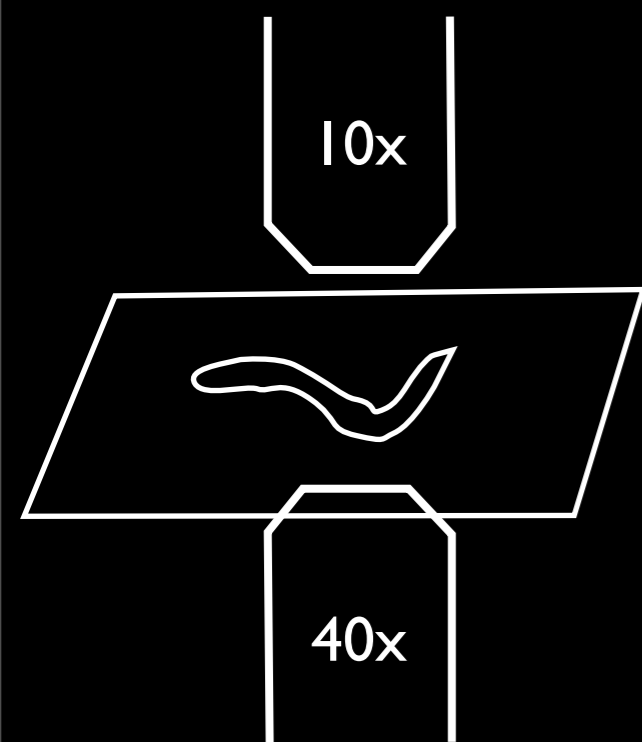
Code:  
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Leifer et al, *Nature Methods* 2012.



# Computer vision based feedback keeps the worm centered over a high magnification objective



# Choosing a real-time tracking methods

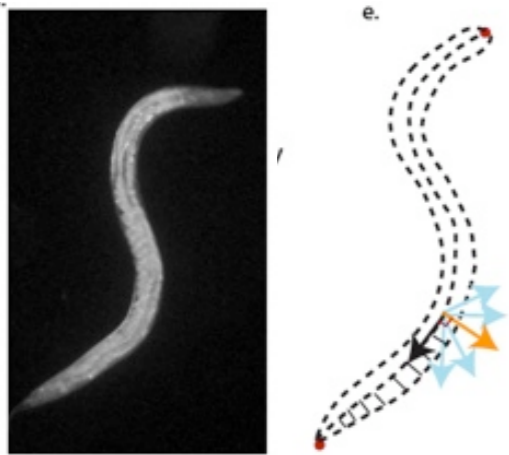


Image processing of bright or darkfield images

Pro	<ul style="list-style-type: none"><li>● Most widely adopted</li><li>● Can track any point</li><li>● Worm body is bright</li><li>● Open source software solutions</li></ul>
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Useful Review: Husson et al., "Keeping Track of Trackers," *WormBook*, 2012.

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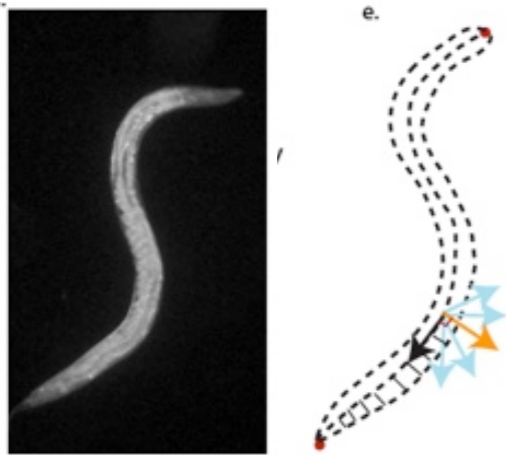


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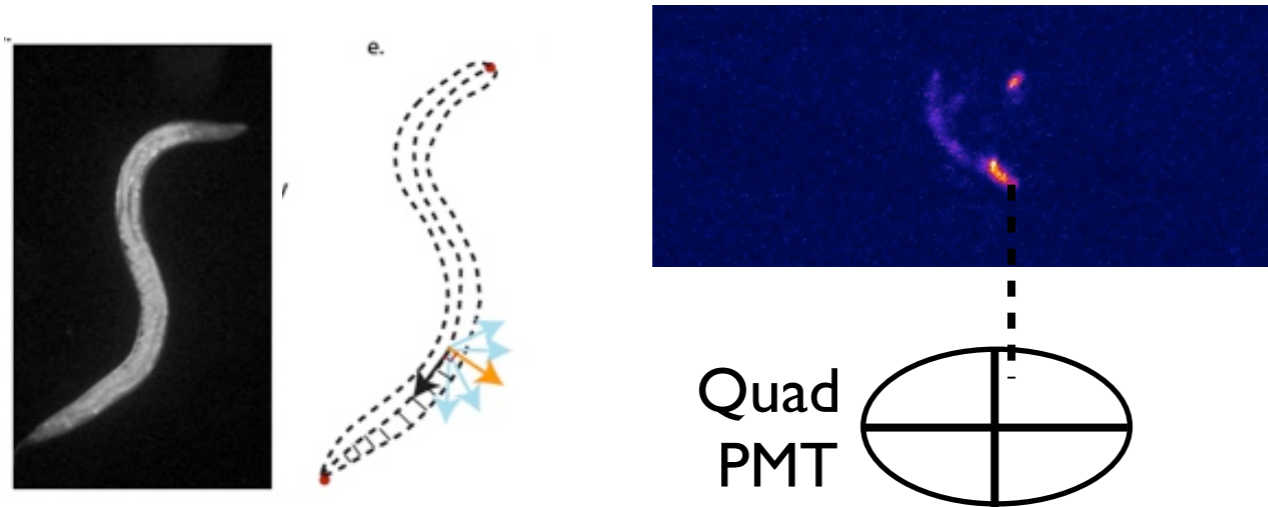


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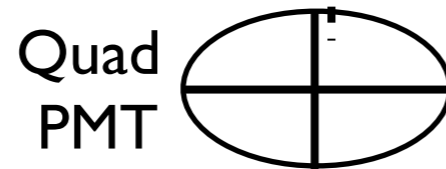
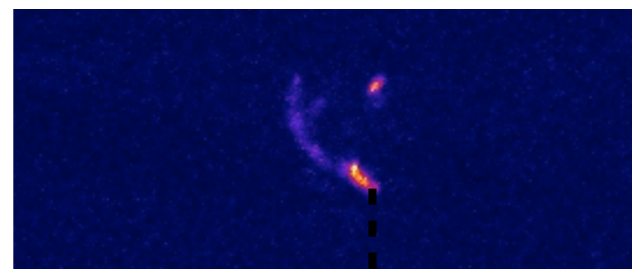
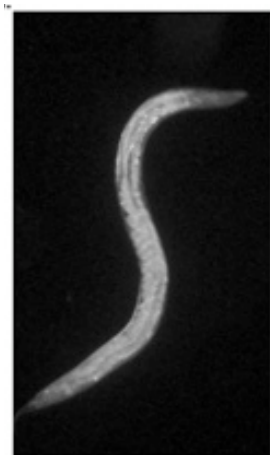


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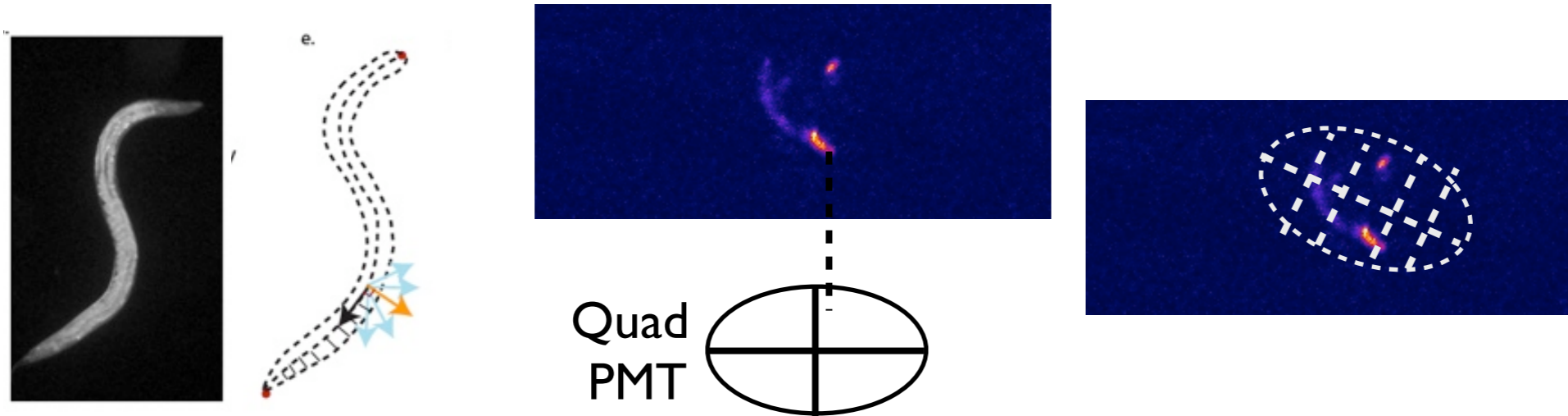


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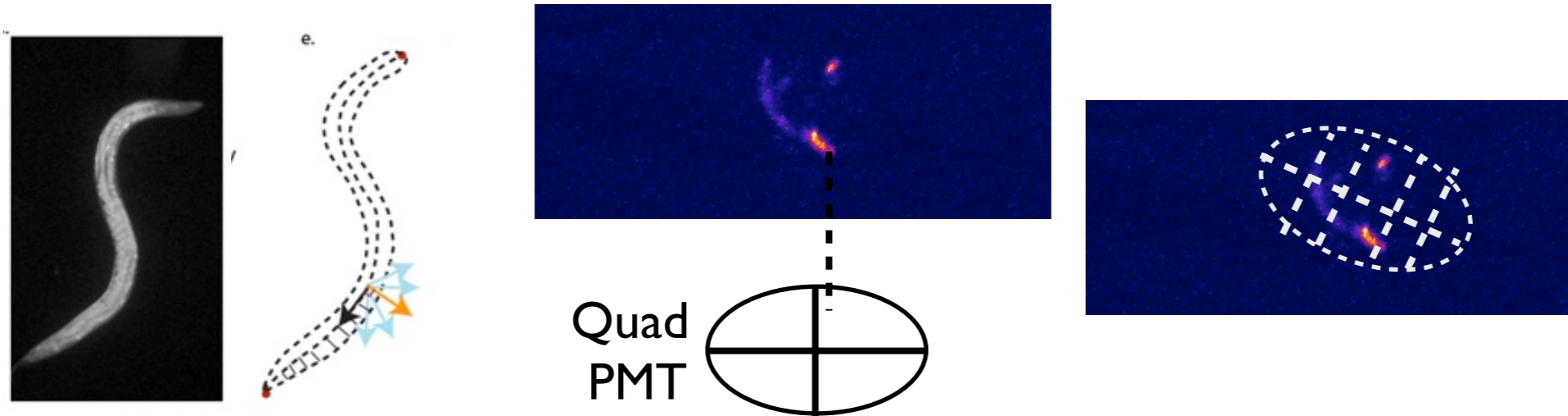


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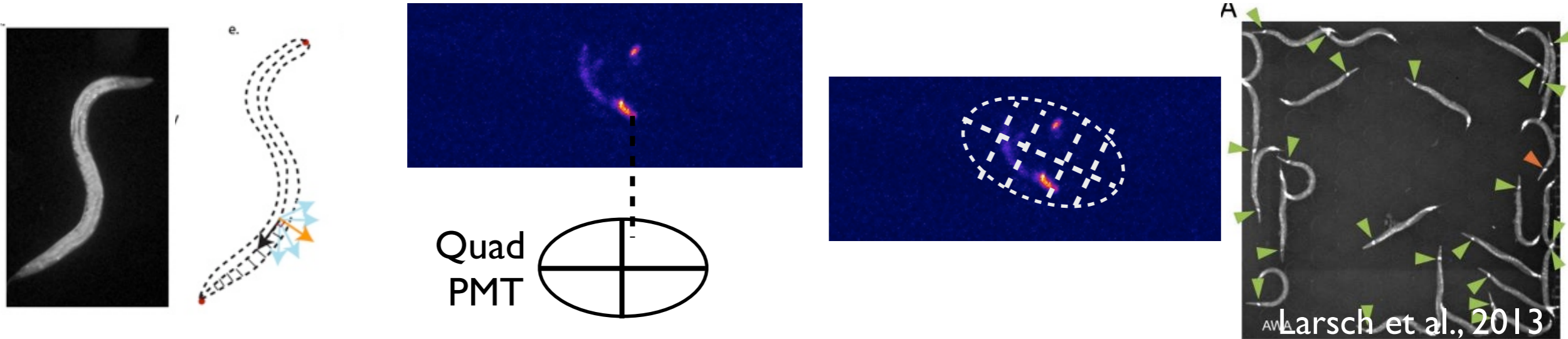


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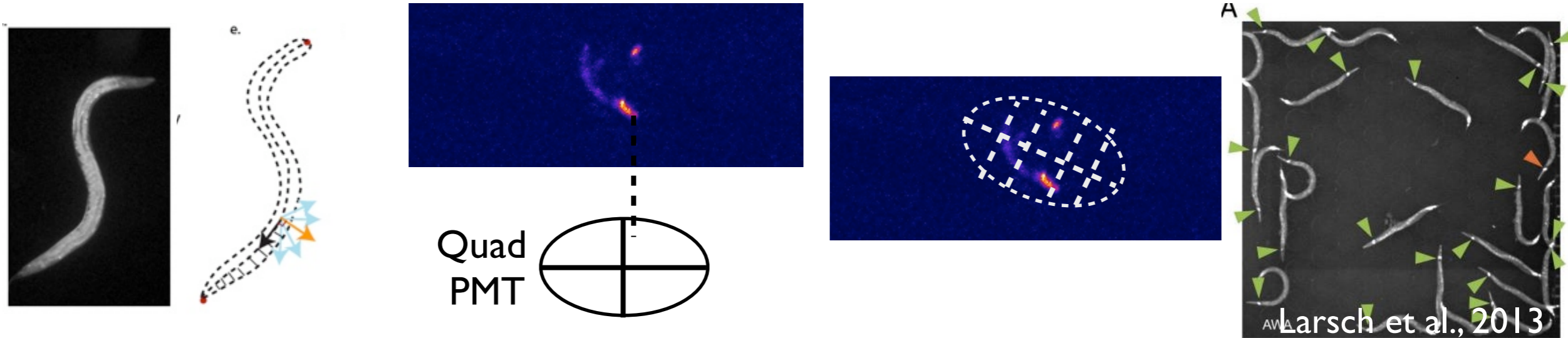
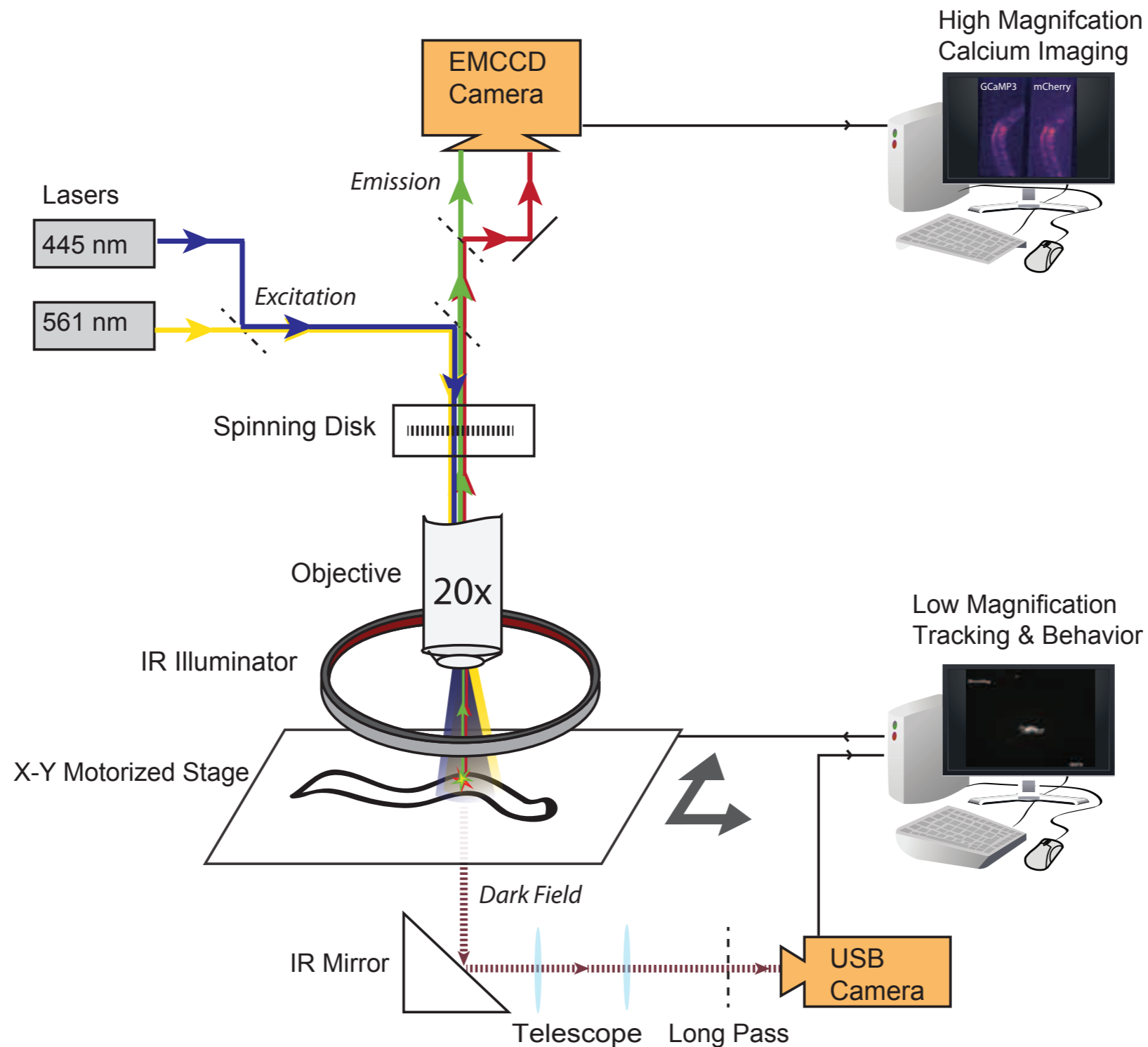


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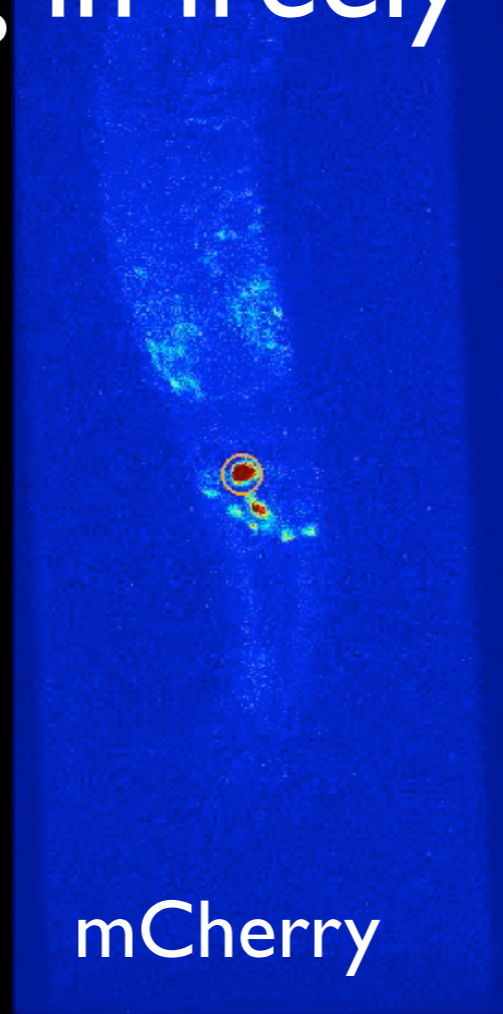
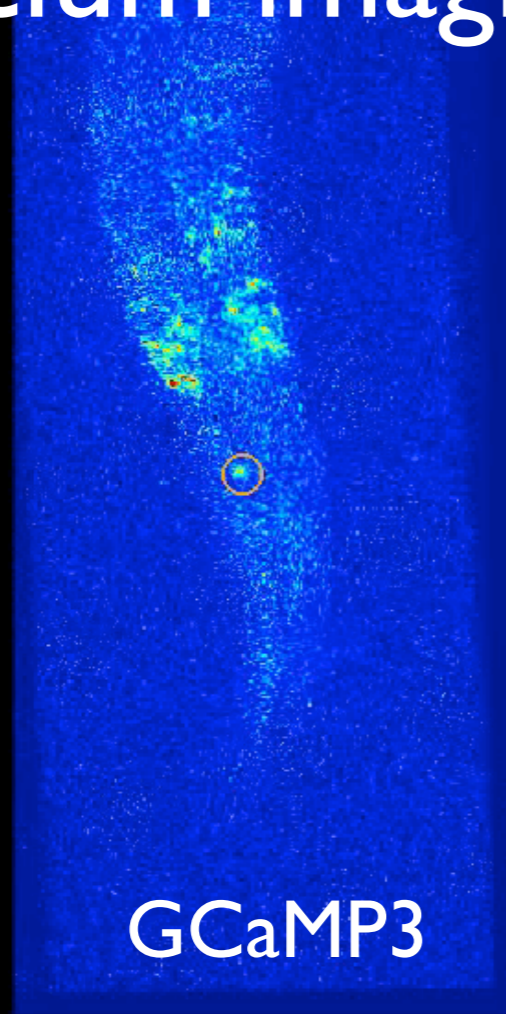
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# Example of freely moving calcium imaging setup

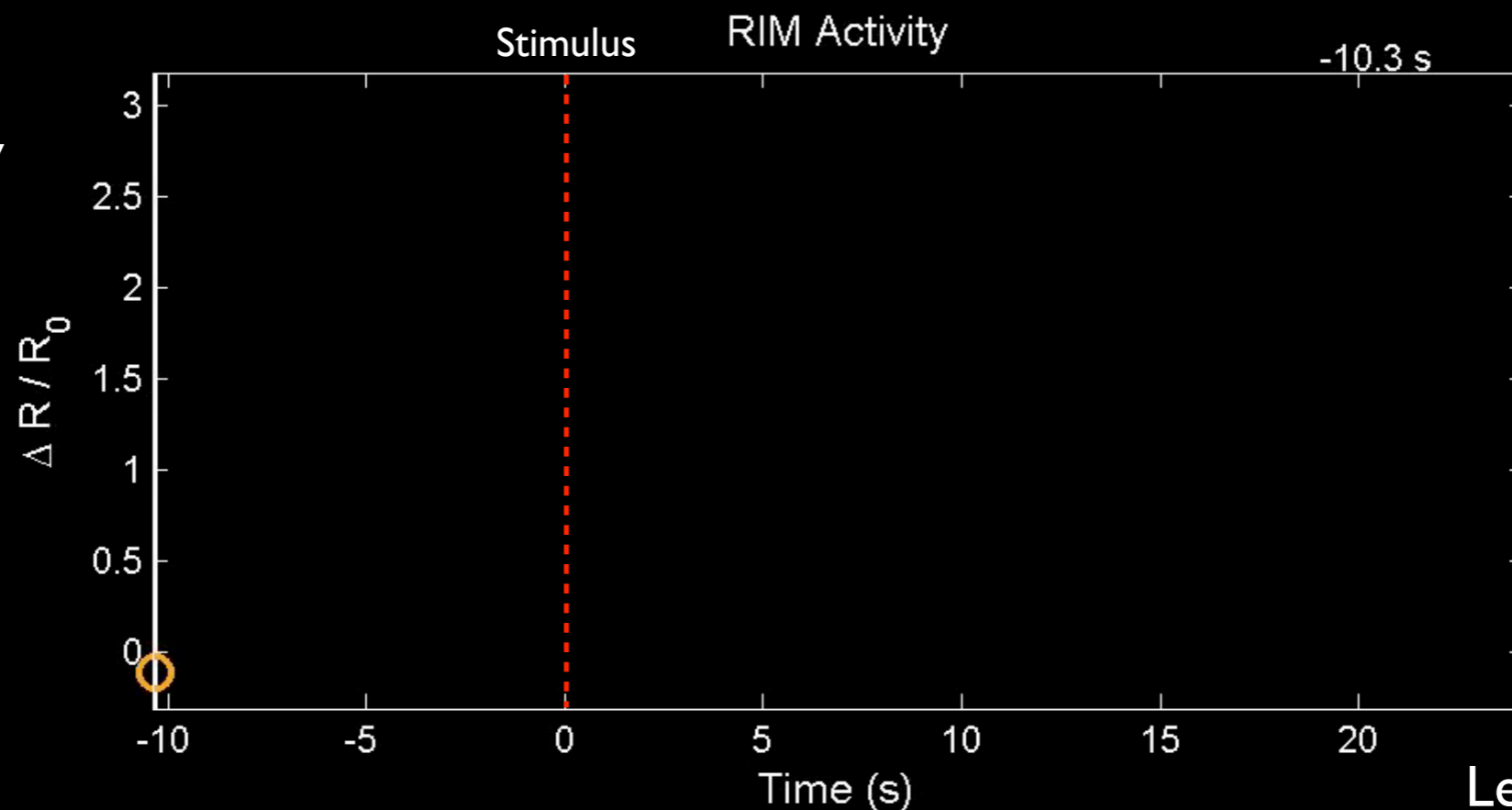


Leifer (thesis) 2012; Leifer & Clark, in prep

# Calcium imaging in freely moving worm



Simultaneously  
measure  
calcium  
transients and  
behavior



Leifer & Clark, in prep

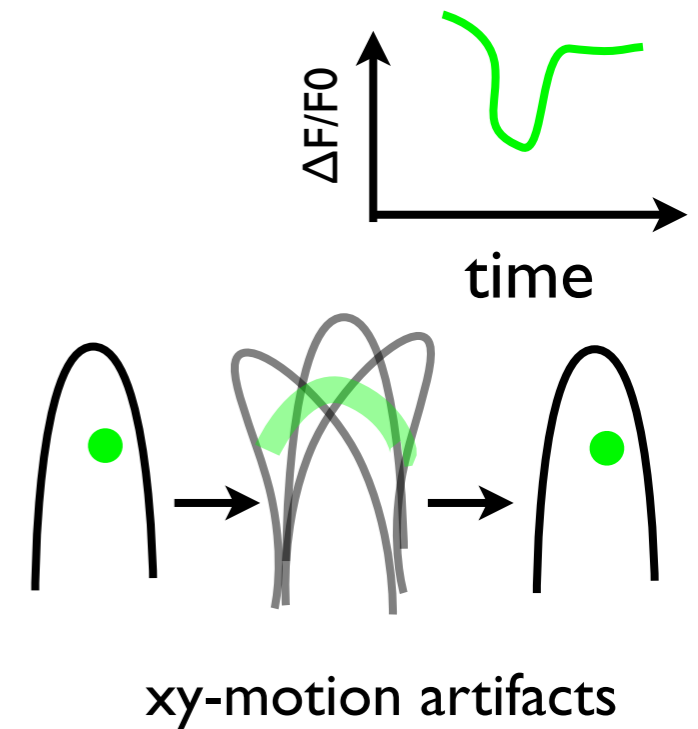
# Common challenges with calcium imaging in freely moving worms

Challenge	Strategy	Solution
Keeping up with the target	Optimize for automated tracking	Large Field of View (Low magnification)
Calcium signal is weak and noisy	Increase signal	Use brightest indicators like GCaMP5k or GCaMP6s
	Collect more photons	Use high NA objectives (high magnification)
	Detect more photons	Use high sensitivity camera CCD: Andor iXon or Photometrics Evolve CMOS: Hamamatsu Orca Flash or Andor Zyla
	Eliminate background fluorescence	<ul style="list-style-type: none"> <li>• Agarose not agar</li> <li>• Spinning disk confocal</li> </ul>
Motion Artifacts Obscure Signal	Use fiducial references	co-express mCherry (or consider true ratiometric indicators)
How to validate	Use controls	Record from GFP instead of GCaMP and ensure your signal is flat

Useful reviews: Kerr, *Wormbook* 2006; Chung et al., 2013;

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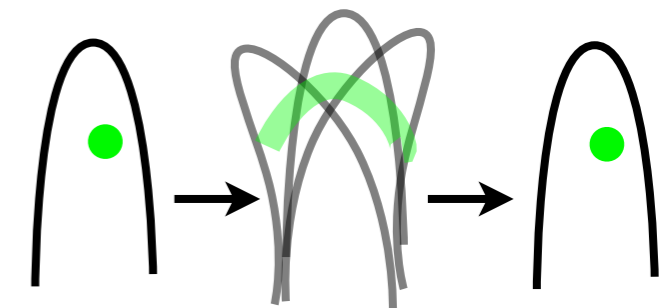
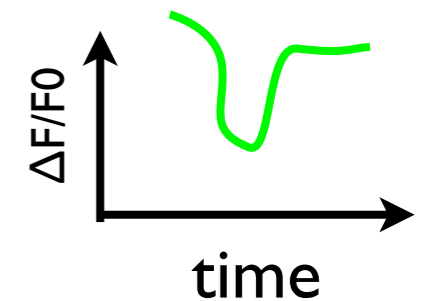
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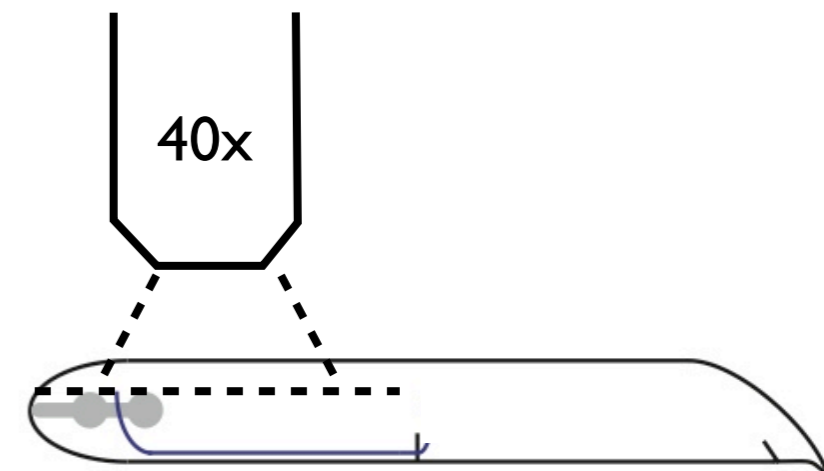
Useful reviews: Kerr, *Wormbook* 2006; Chung et al., 2013;

# Common challenges with calcium imaging in freely moving worms

Challenge	Strategy	Solution
Keeping up with the target	Optimize for automated tracking	Large Field of View (Low magnification)
Calcium signal is weak and noisy	Increase signal	Use brightest indicators like GCaMP5k or GCaMP6s
	Collect more photons	Use high NA objectives (high magnification)
	Detect more photons	Use high sensitivity camera CCD: Andor iXon or Photometrics Evolve CMOS: Hamamatsu Orca Flash or Andor Zyla
	Eliminate background fluorescence	<ul style="list-style-type: none"> <li>Agarose not agar</li> <li>Spinning disk confocal</li> </ul>
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xy-motion artifacts

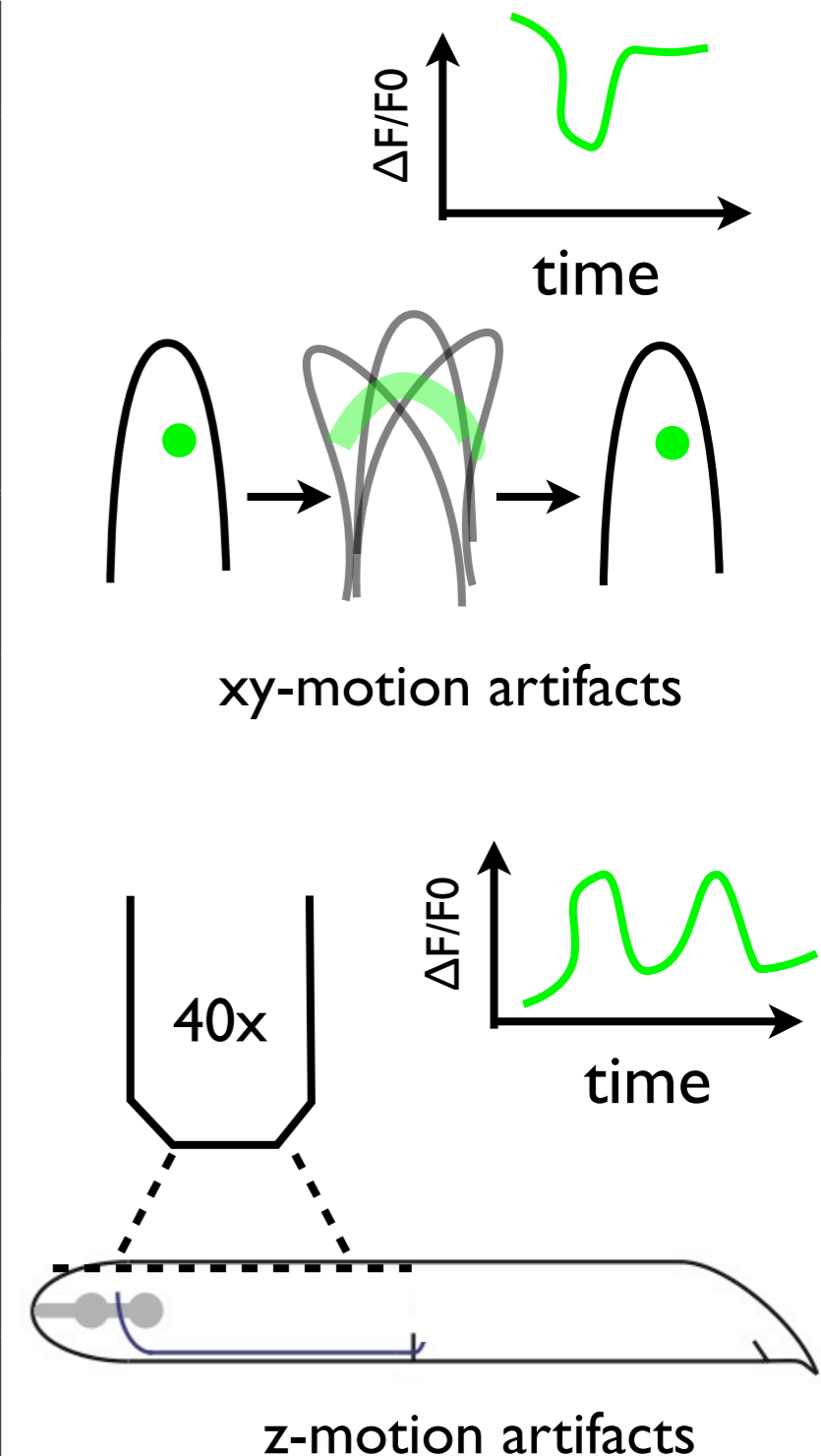


z-motion artifacts

Useful reviews: Kerr, *Wormbook* 2006; Chung et al., 2013;

# Common challenges with calcium imaging in freely moving worms

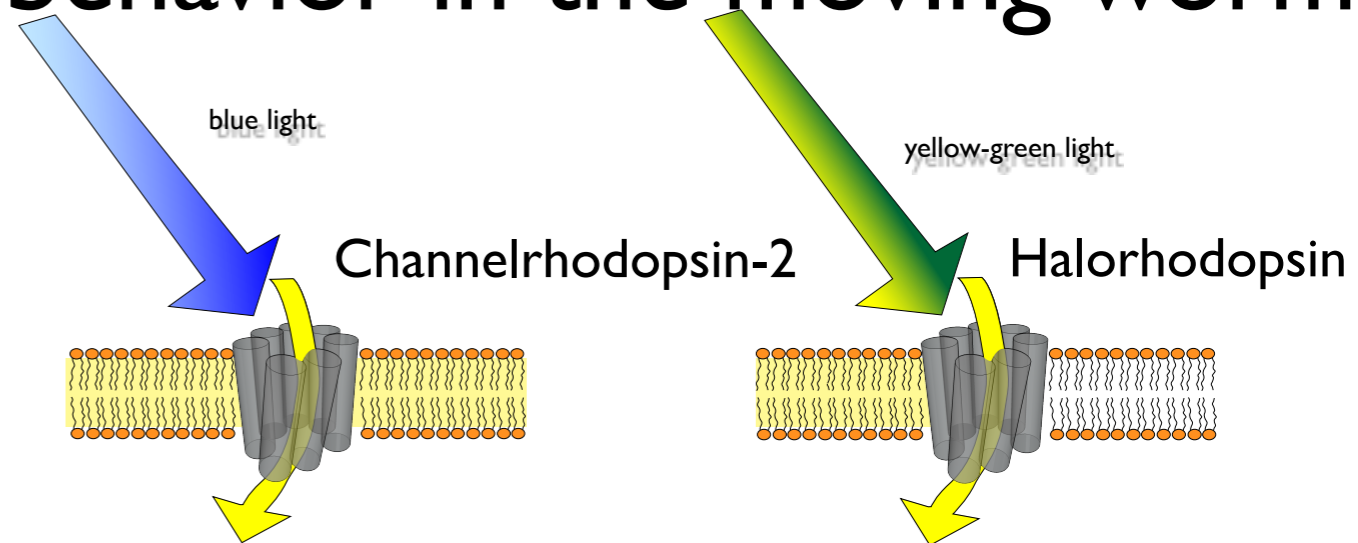
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# Optogenetic manipulation of behavior in the moving worm

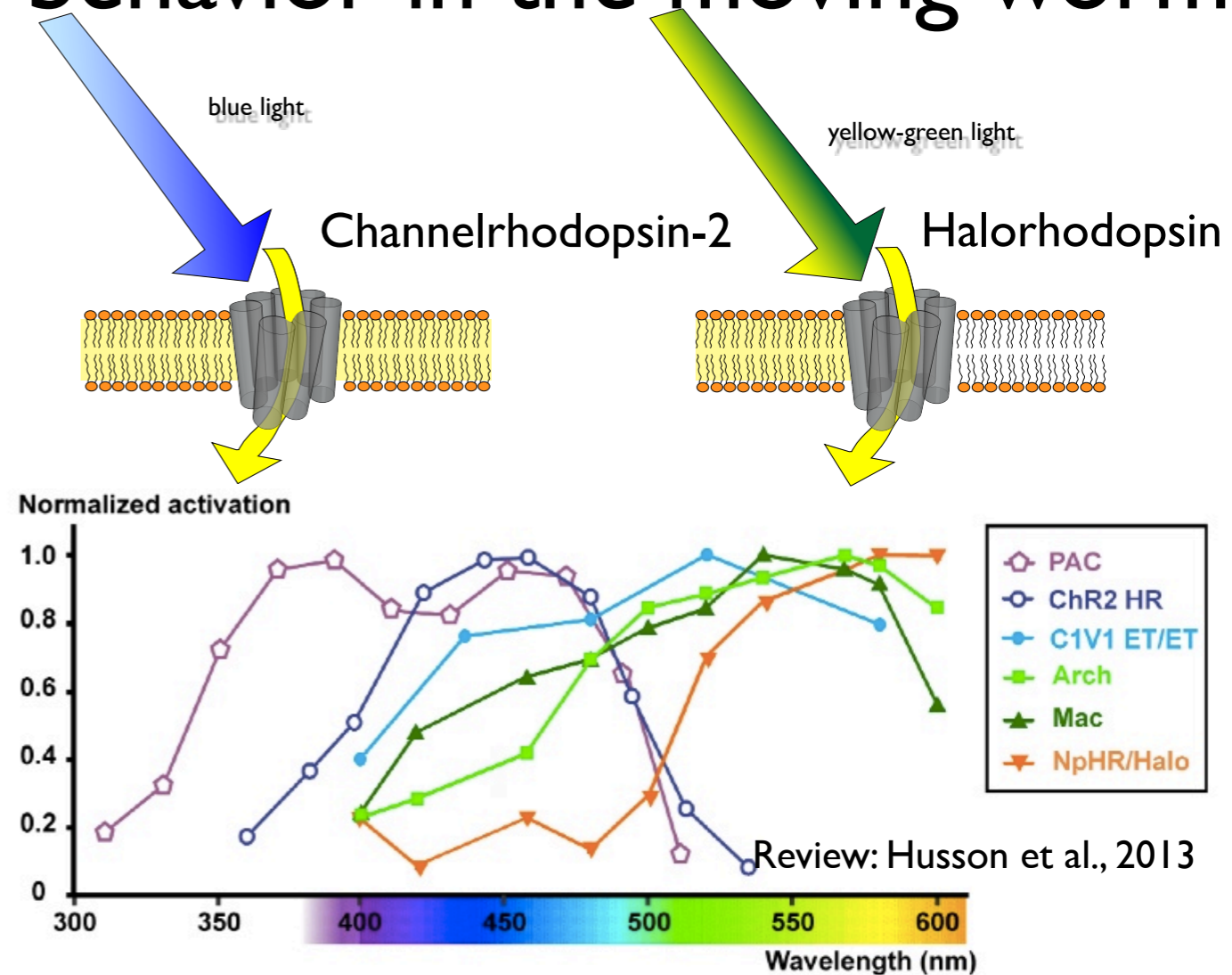
- First optogenetic manipulations of behavior by ChR2 in any animal occurred in free moving *C. elegans* (Nagel et al., 2005)
- There is an ever-growing toolbox of optogenetic proteins tested in worms
- Previously, ability to target individual neurons was limited by genetic promotor
- Targeted illumination systems first in immobilized worms ( Guo et al., 2009 ) and now in moving worms can provide single cell specificity





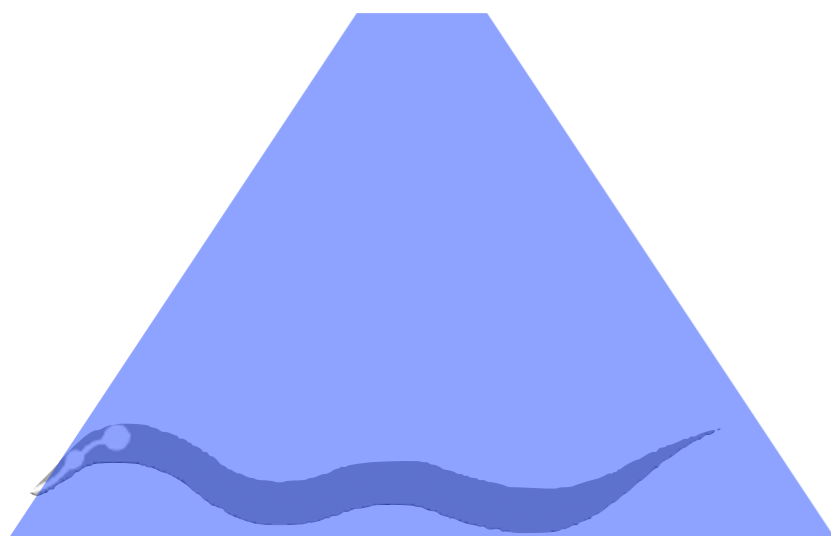
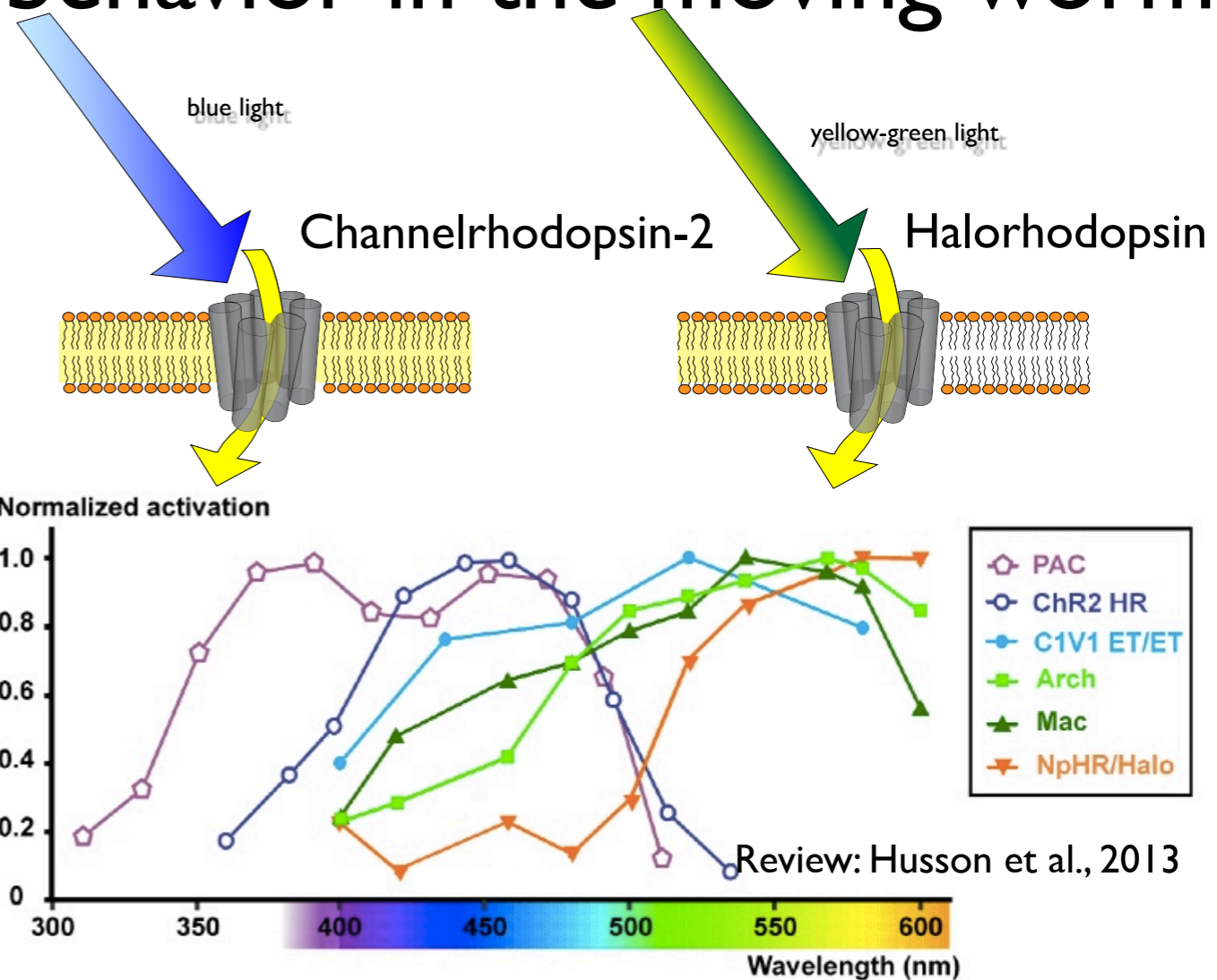
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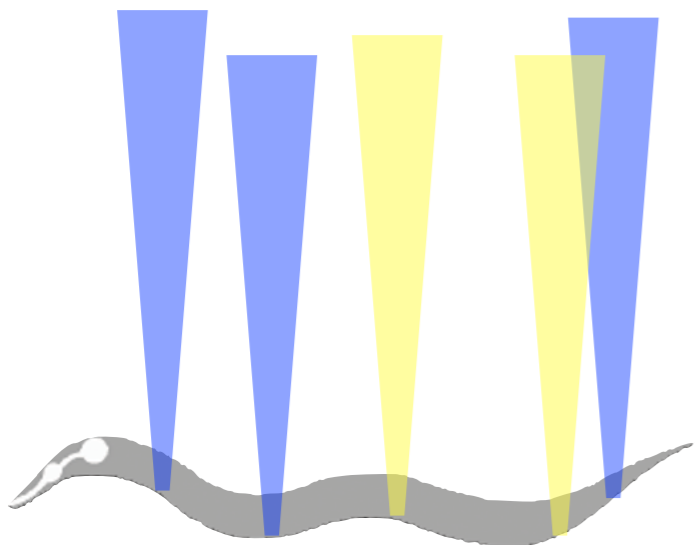
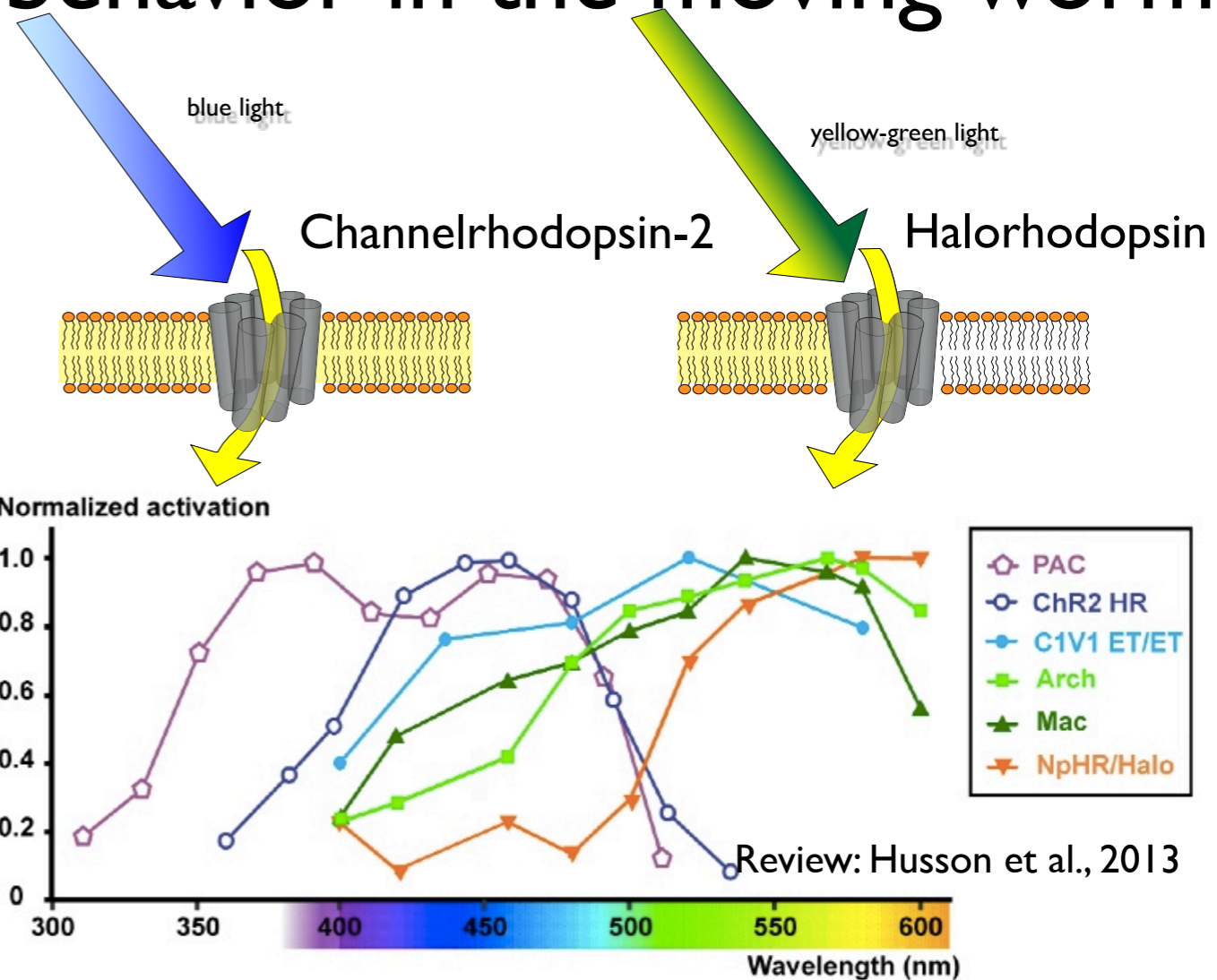
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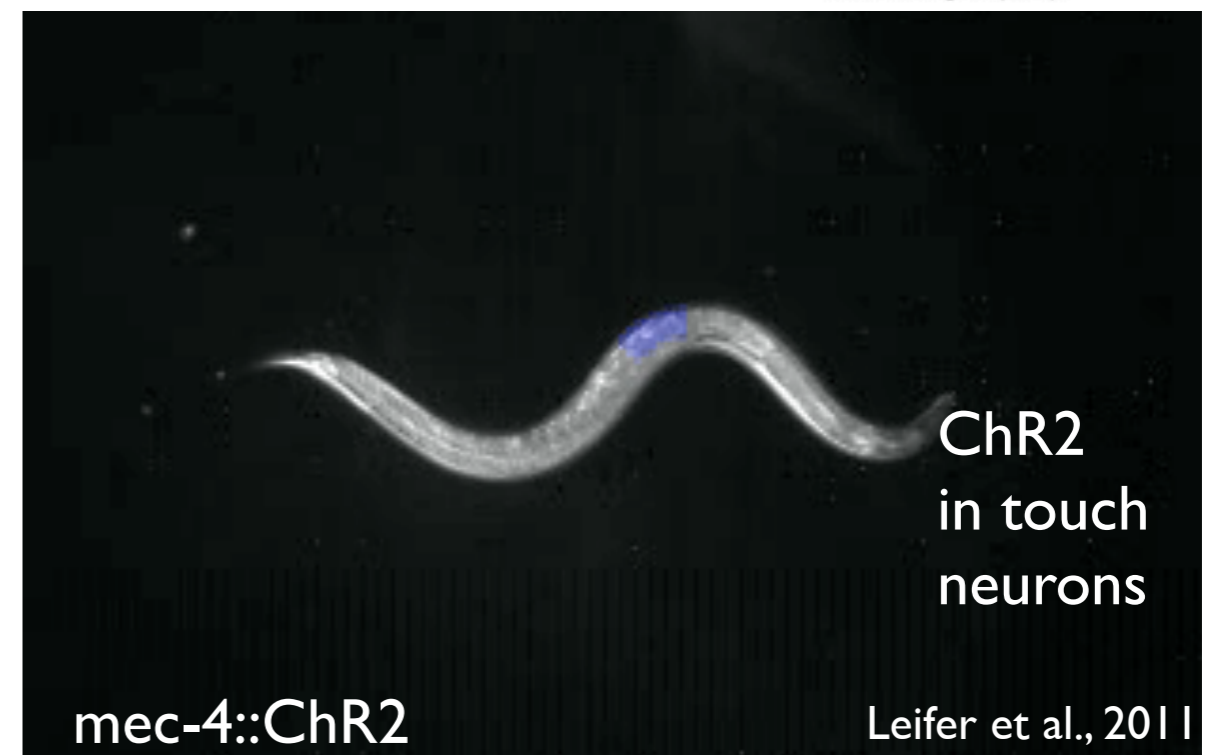
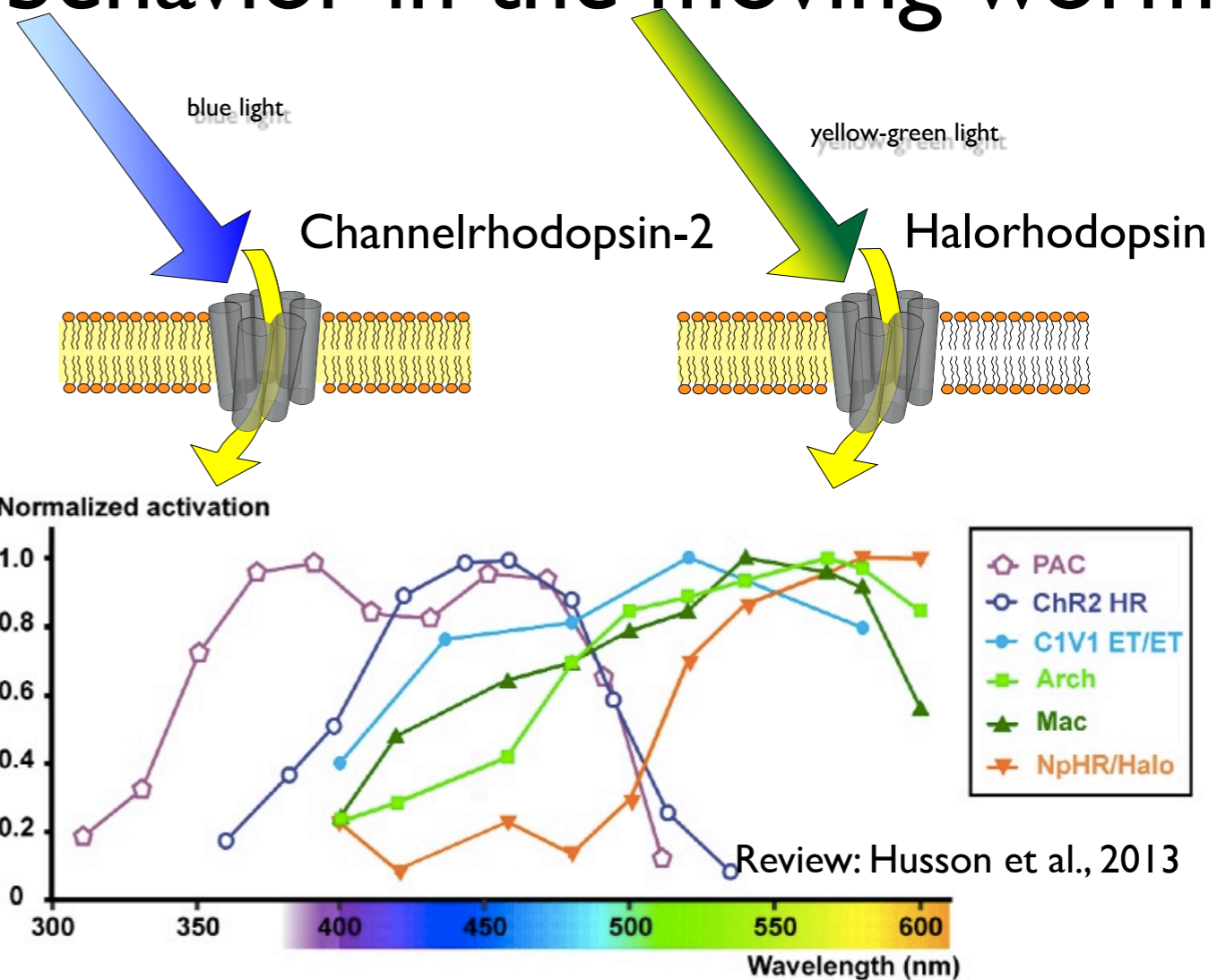
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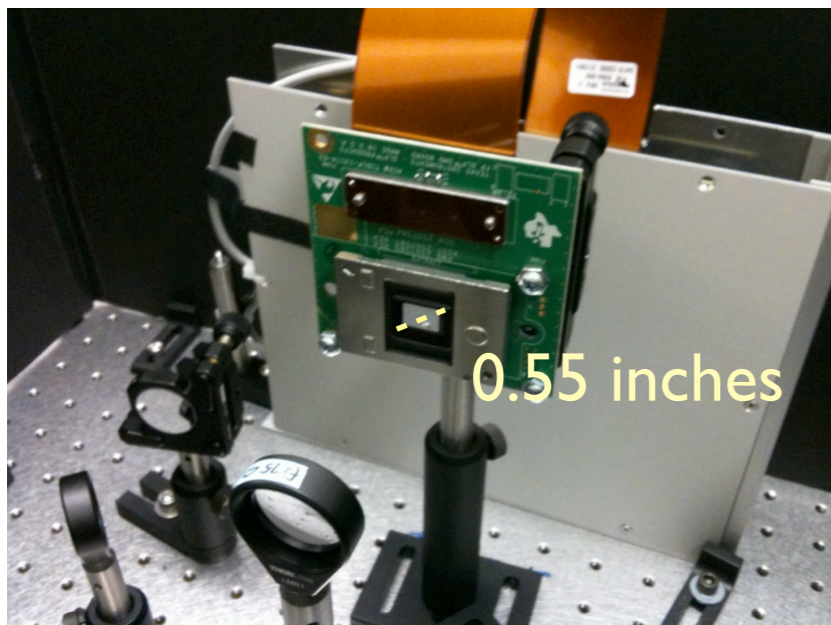
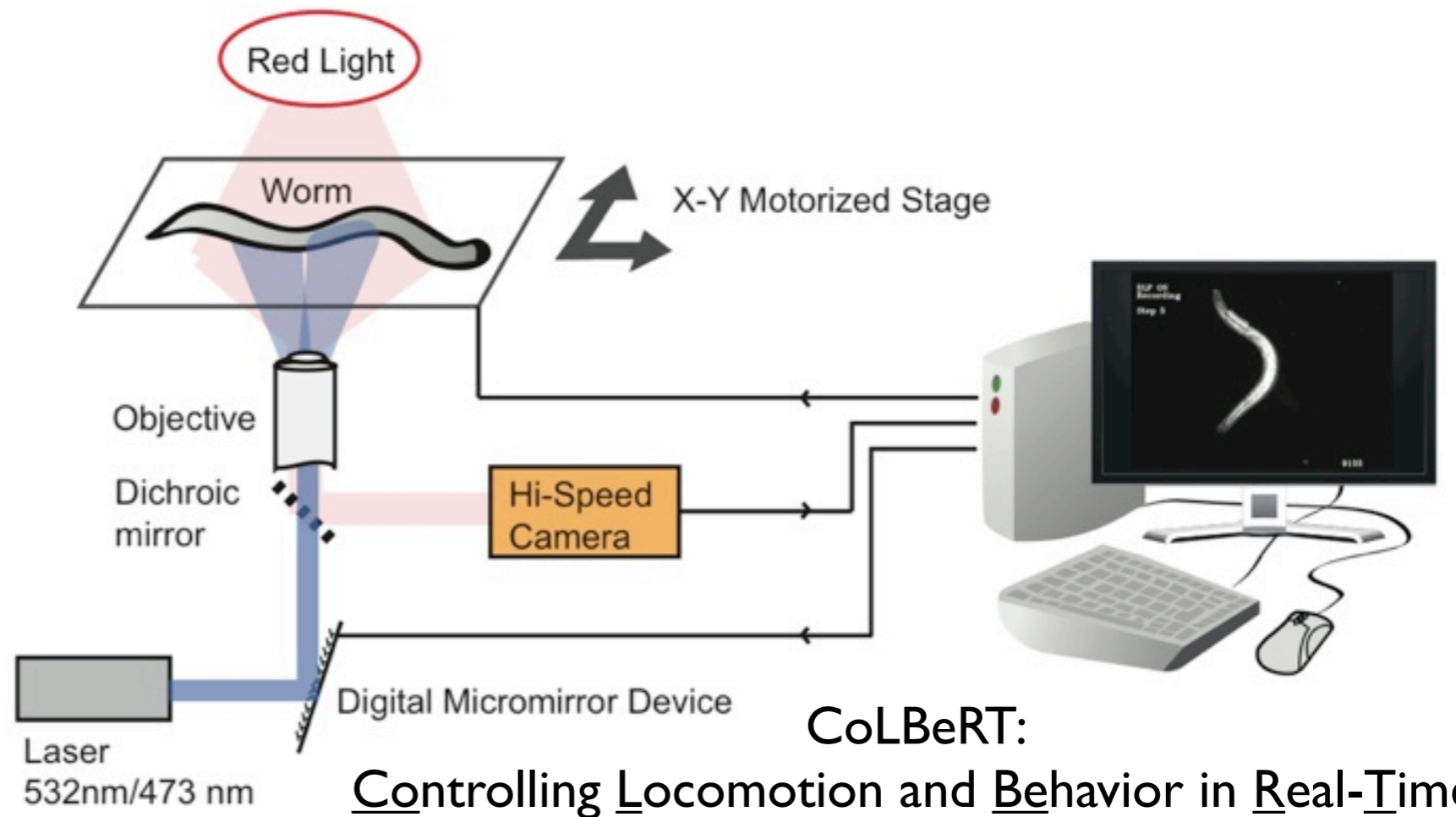
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# Targeted illumination systems in freely moving worm

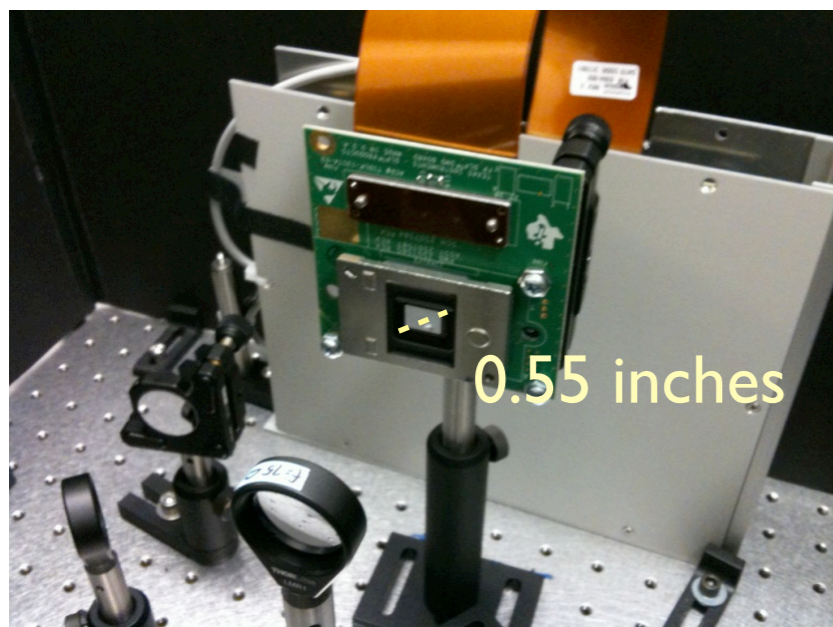
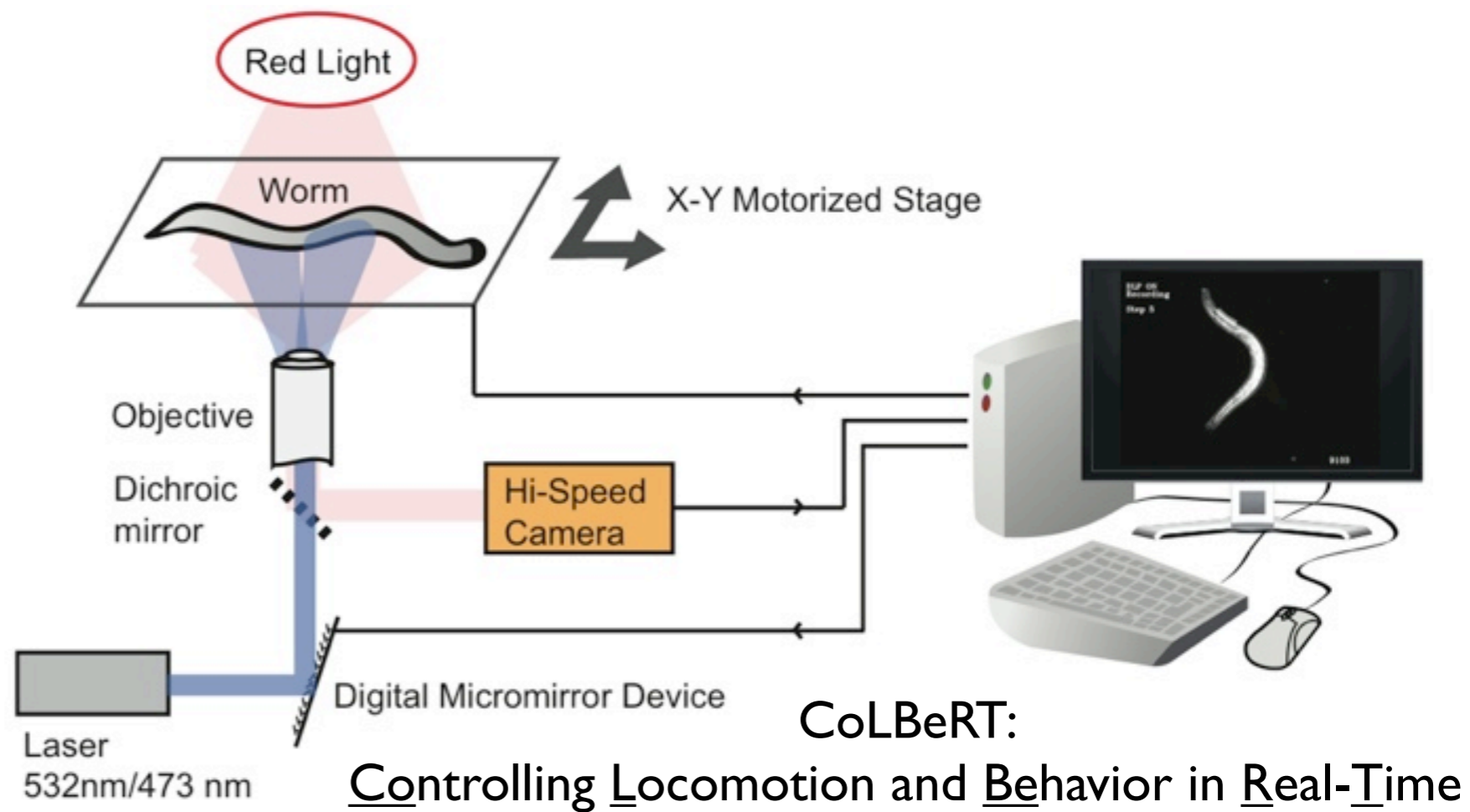
- One of two systems developed independently (Leifer et al, 2011; Stirman et al 2011)
- 80 Hz
- Round trip latency of 28 ms



700,000 mirrors

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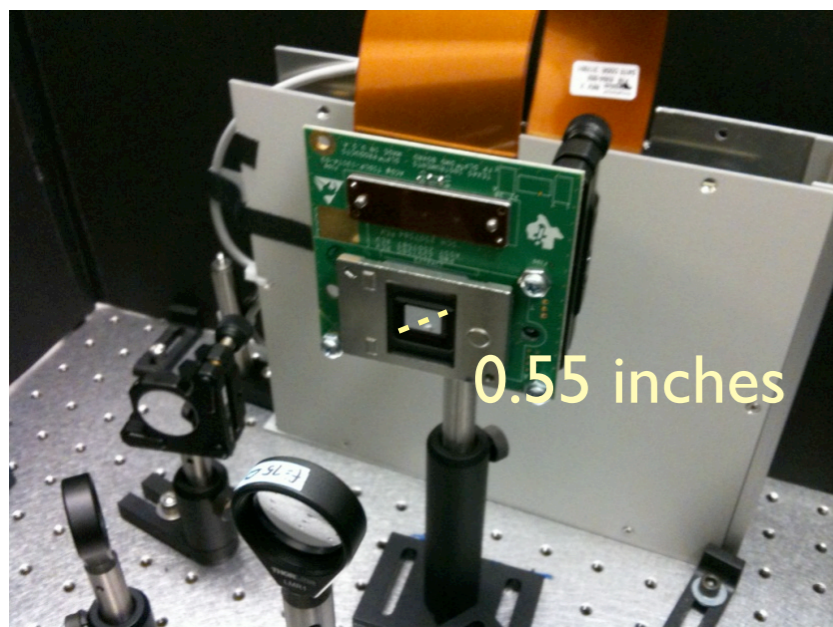
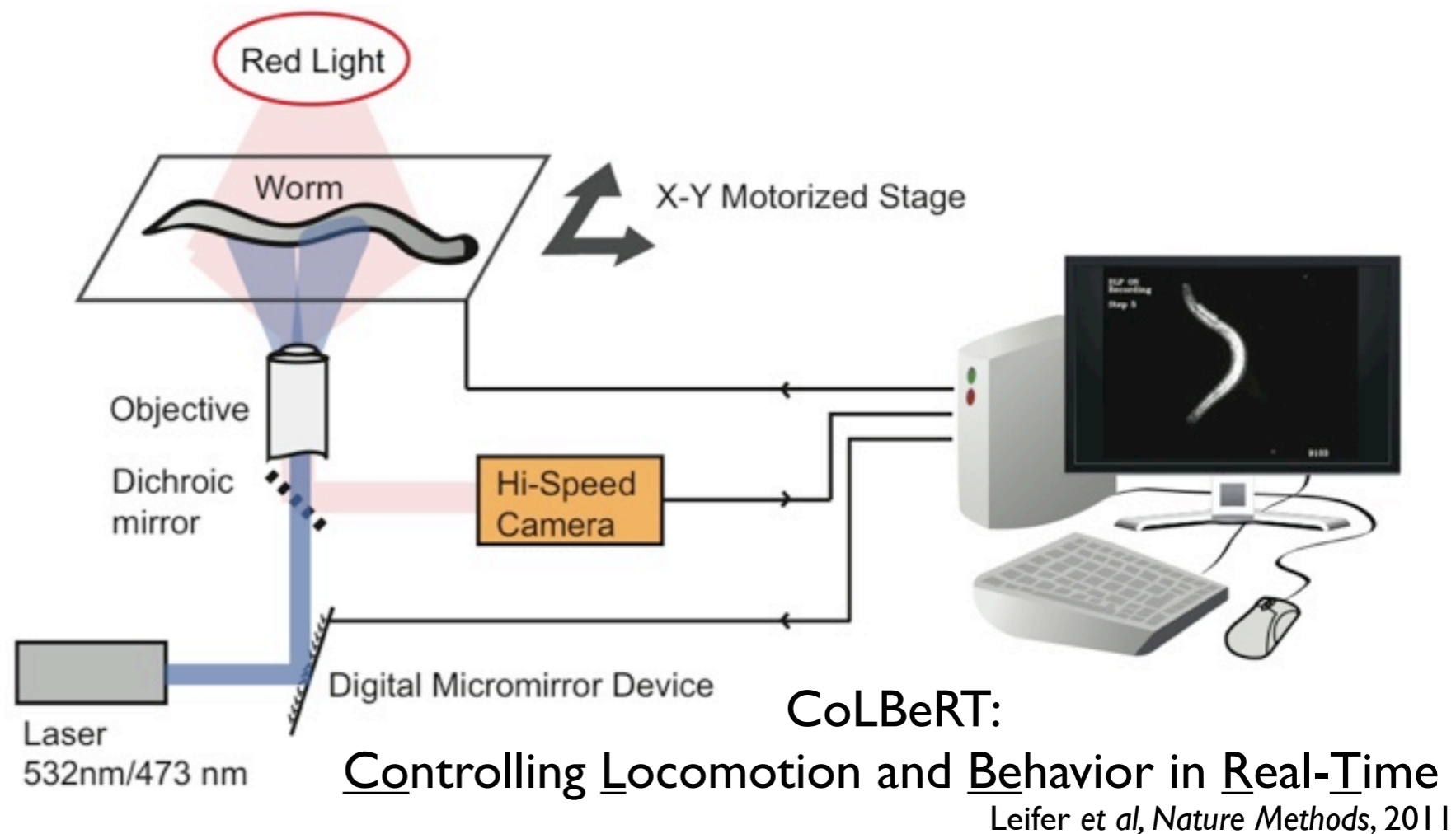
Digital micromirror device  
700,000 mirrors



Outline-based targeting

# Targeted illumination systems in freely moving worm

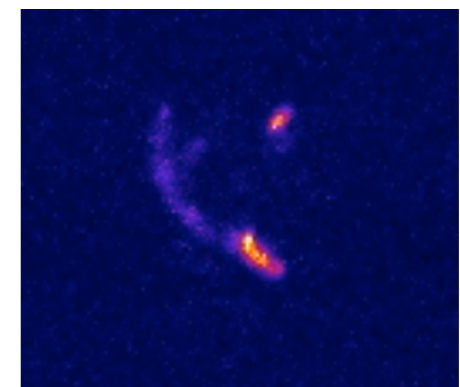
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Digital micromirror device  
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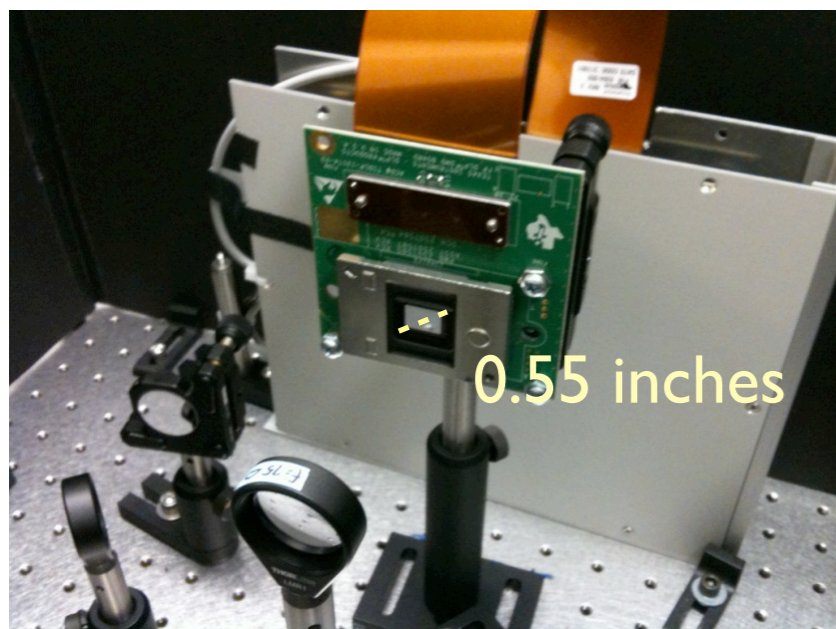
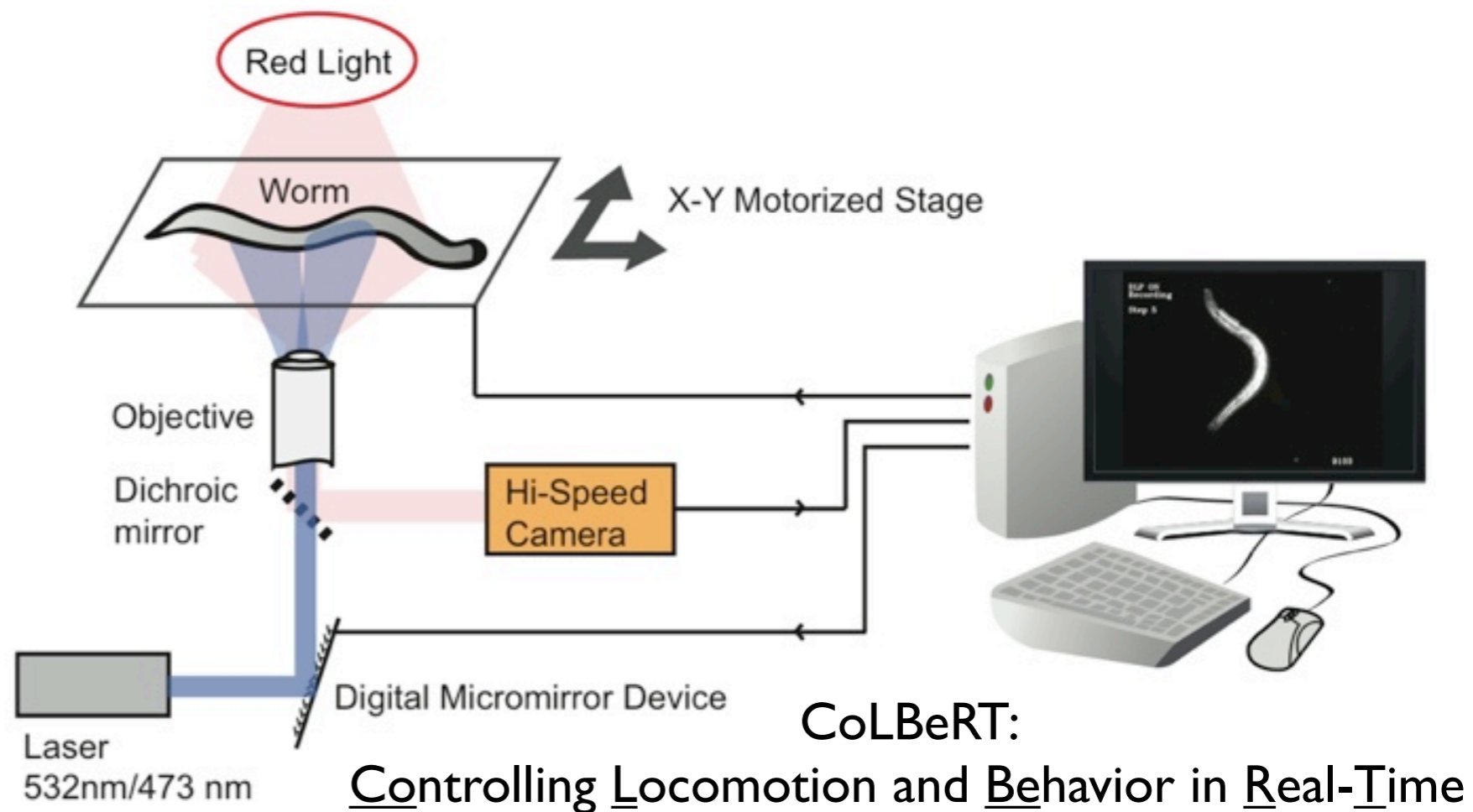
Outline-based targeting



Fluorescence targeting  
Kocabas et al., 2012

# Targeted illumination systems in freely moving worm

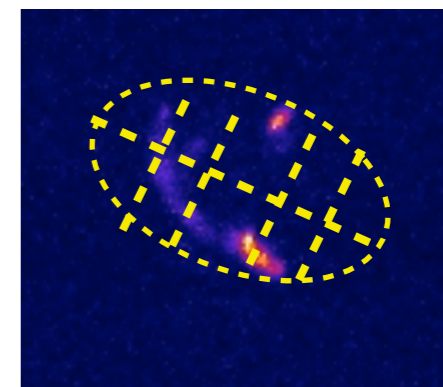
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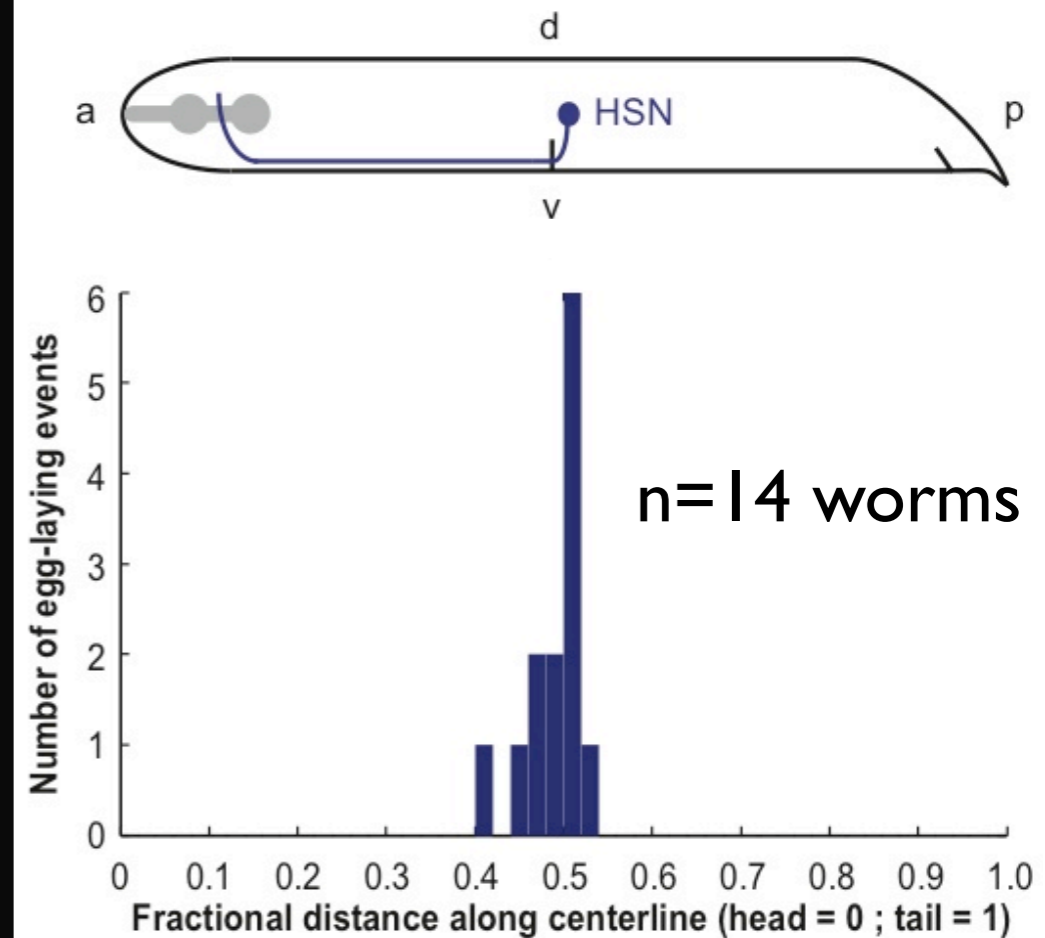
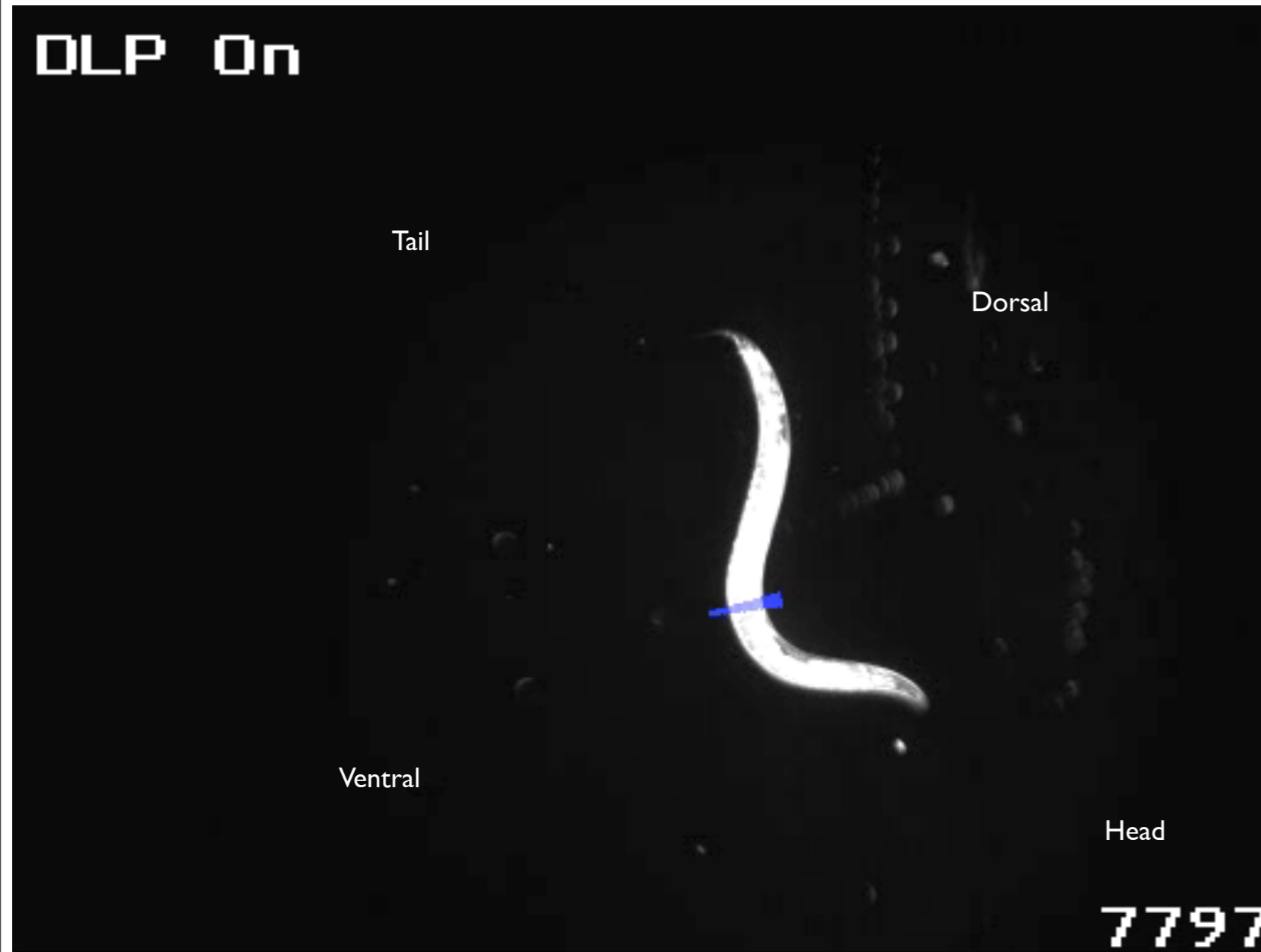
Outline-based targeting



Fluorescence targeting  
Kocabas et al., 2012



# CoLBeRT has anterior-posterior accuracy



~30 micron reproducibility





Channelrhodopsin in  
egg-laying **motoneuron**

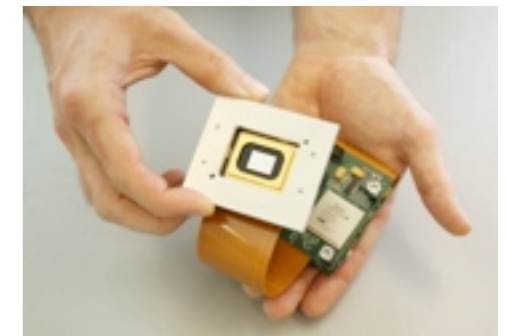
*CoLBeRT can reproducibly target a single motor neuron*

*Pegl-6::ChR2::YFP*  
Gift of N. Ringstad

Leifer et al, *Nature Methods*, 2011

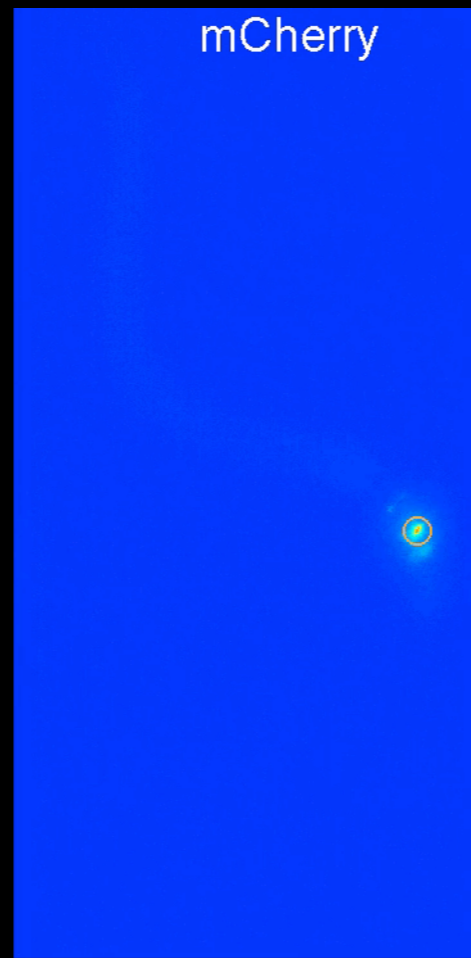
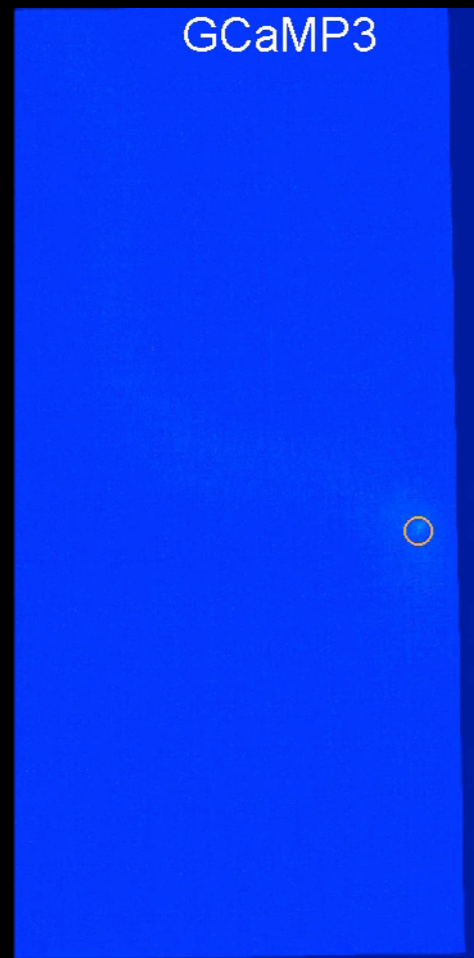
# Patterned illumination systems

	Vendor / Model	Software for real-time <i>C. elegans</i> targeting	References
<b>Off the Shelf Projector</b>	Hitachi	open source (LabView)	Stirman et al., 2011 Stirman et al., 2012
<b>Build from components</b>		open source (C) <a href="http://git.io/colbert">http://git.io/colbert</a>	Leifer et al., 2011
<b>Pre-built</b>	  	none publicly available	Kocabas et al, 2012; N/A N/A

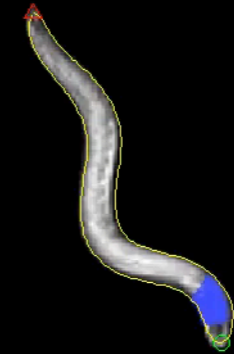


- Projector is well documented and cost effective but latency can be problematic
- Commercial systems will require software development

# Combining calcium imaging and optogenetics in the moving worm

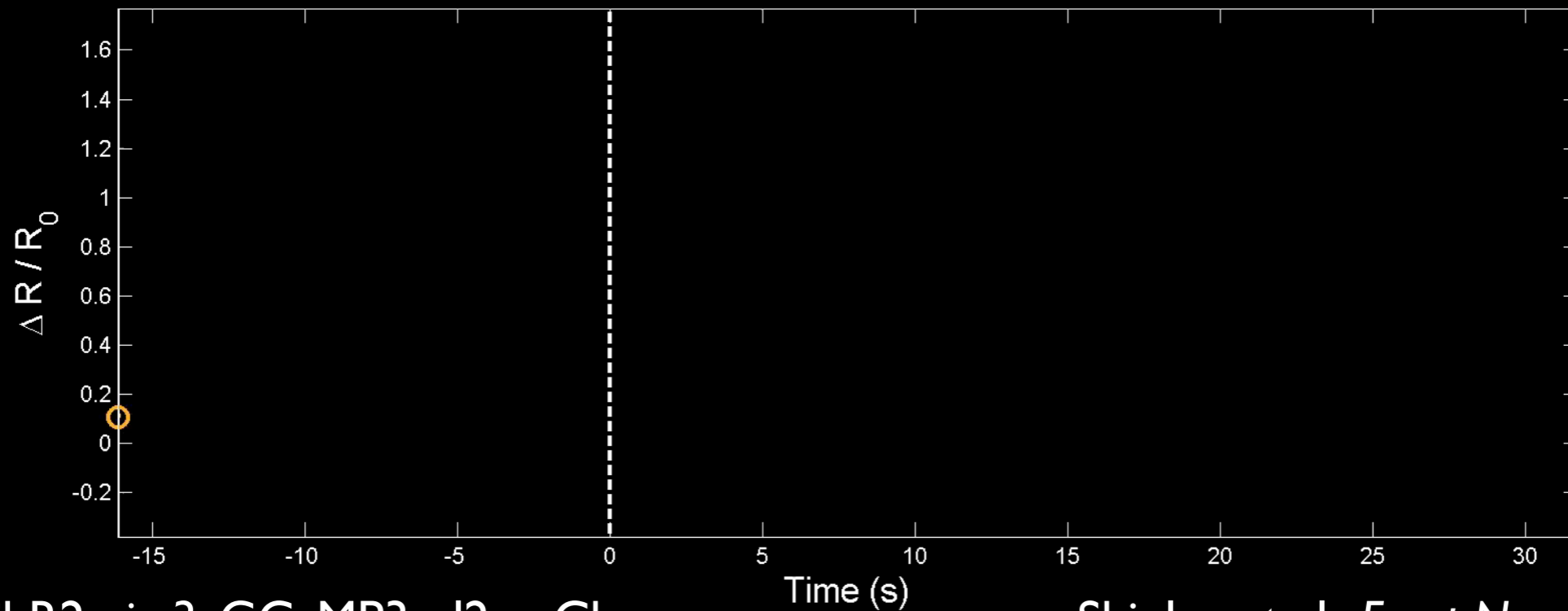


Behavior



AVA Activity

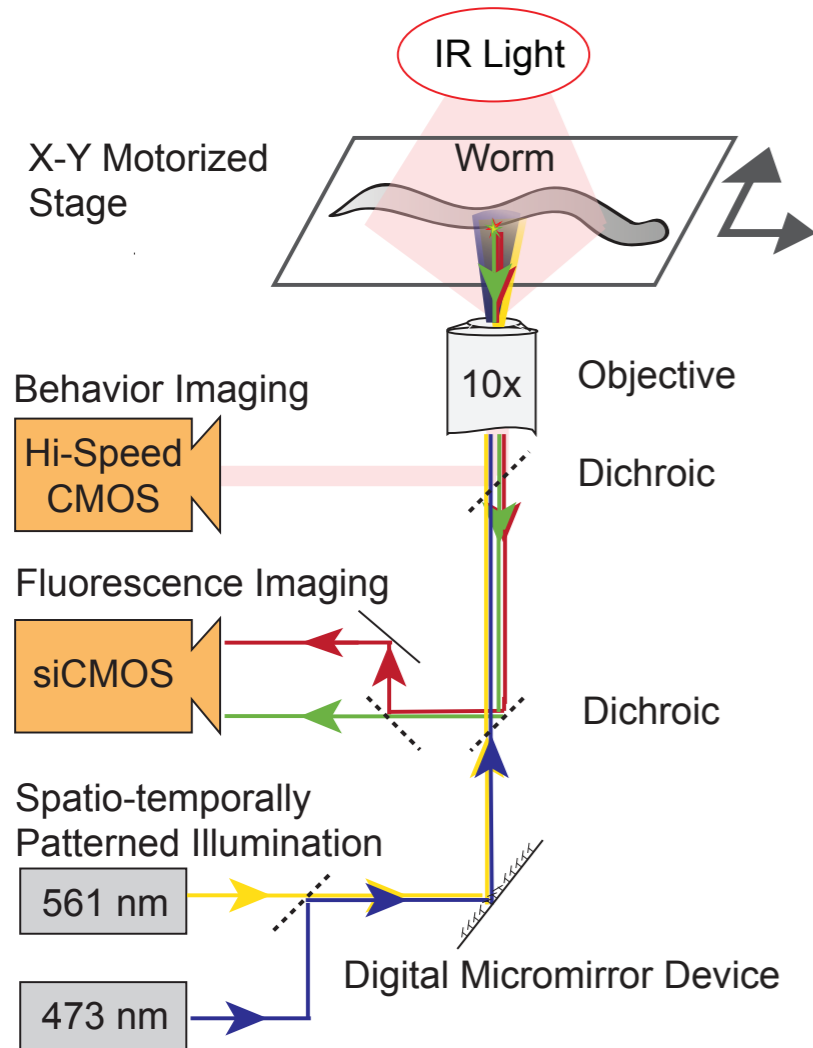
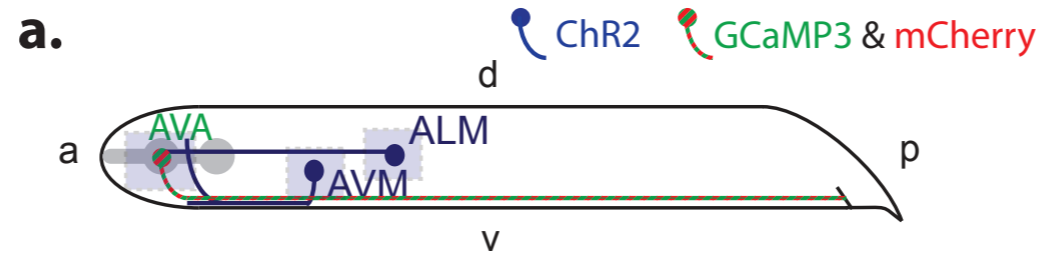
-16.1 s



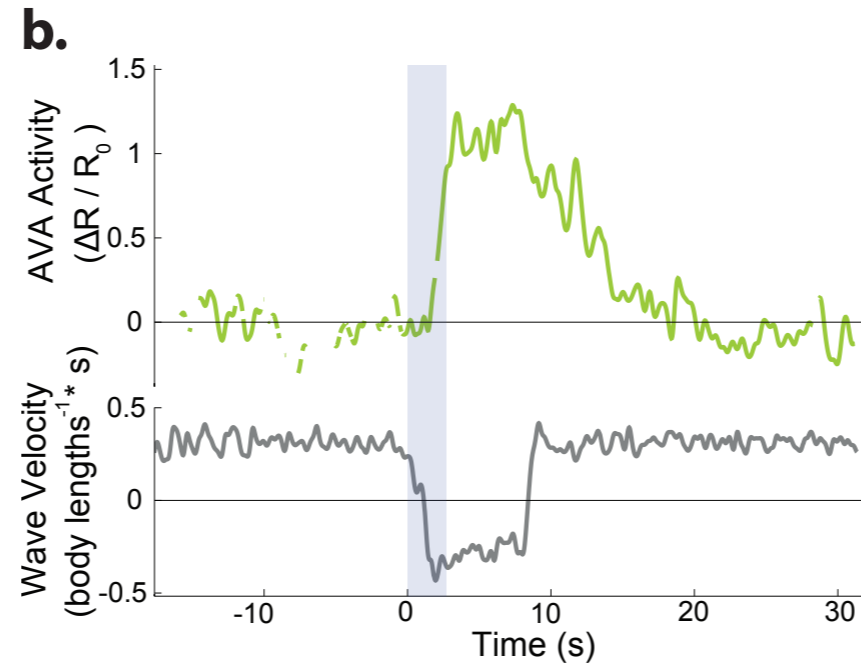
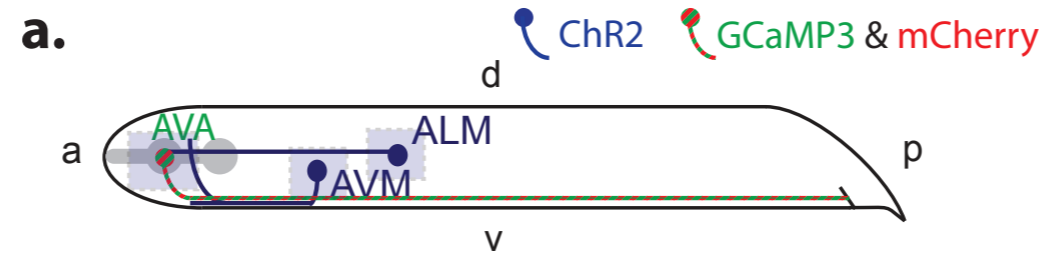
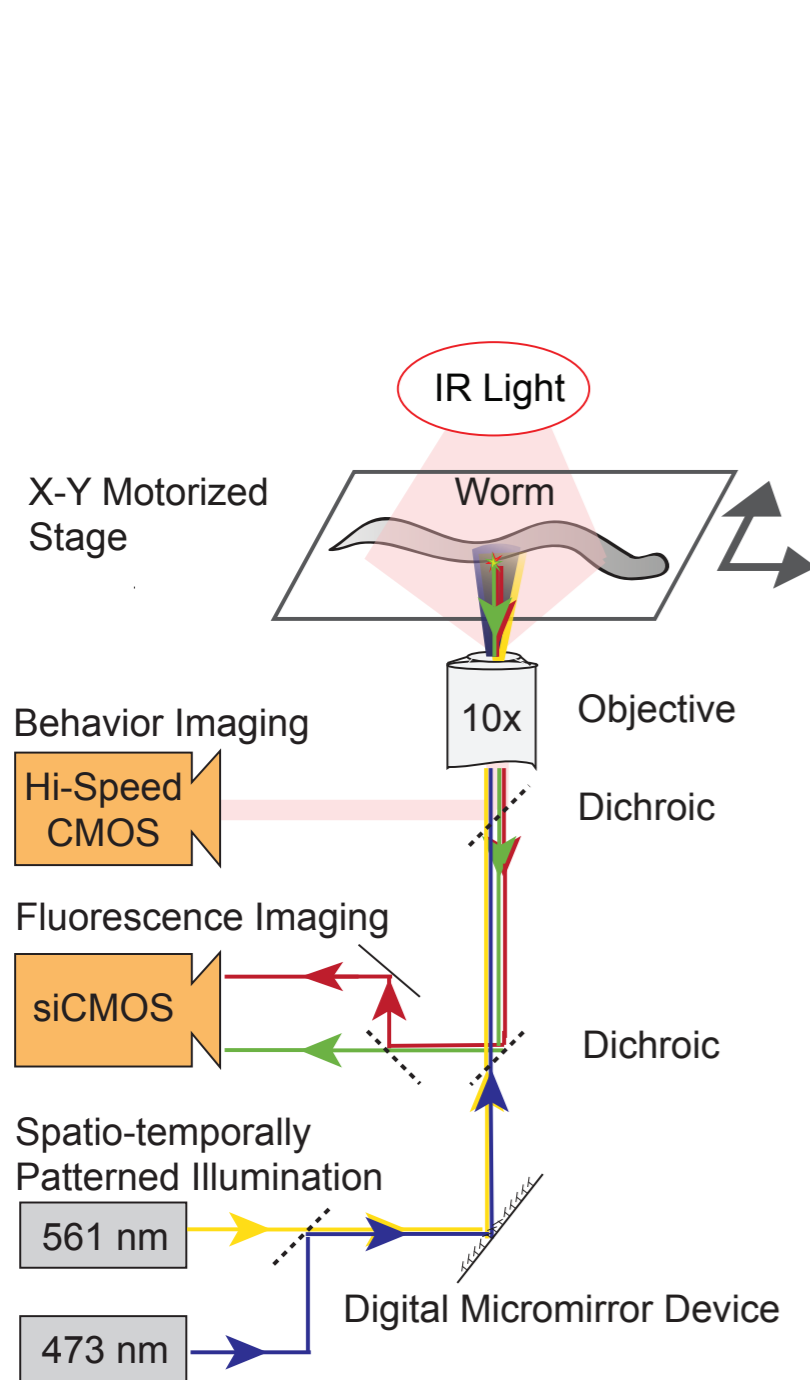
*mec-4::ChR2, rig-3::GCaMP3::sl2::mCherry*

Shiple et al., *Front Neural Circuits*, 2014

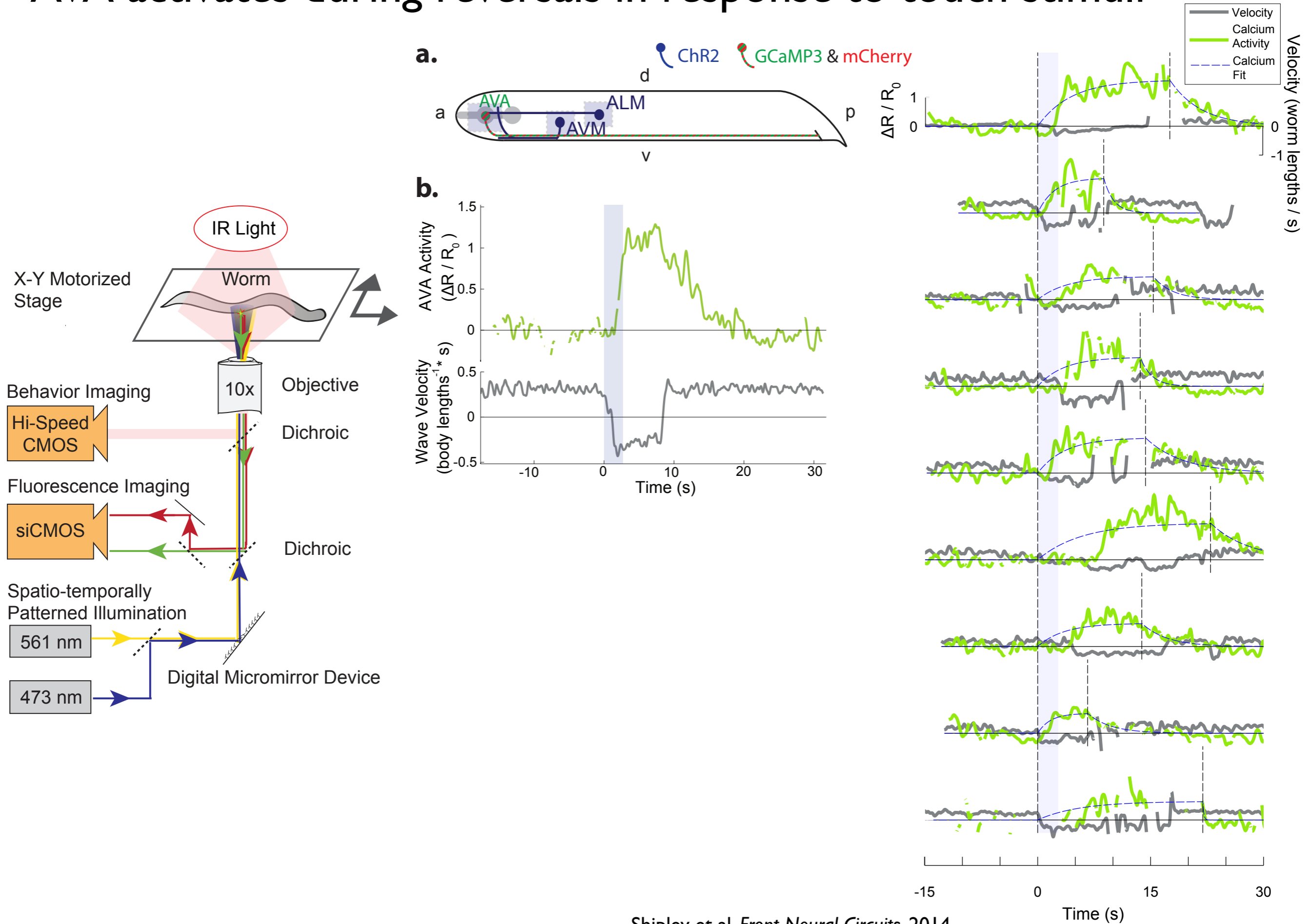
# AVA activates during reversals in response to touch stimuli



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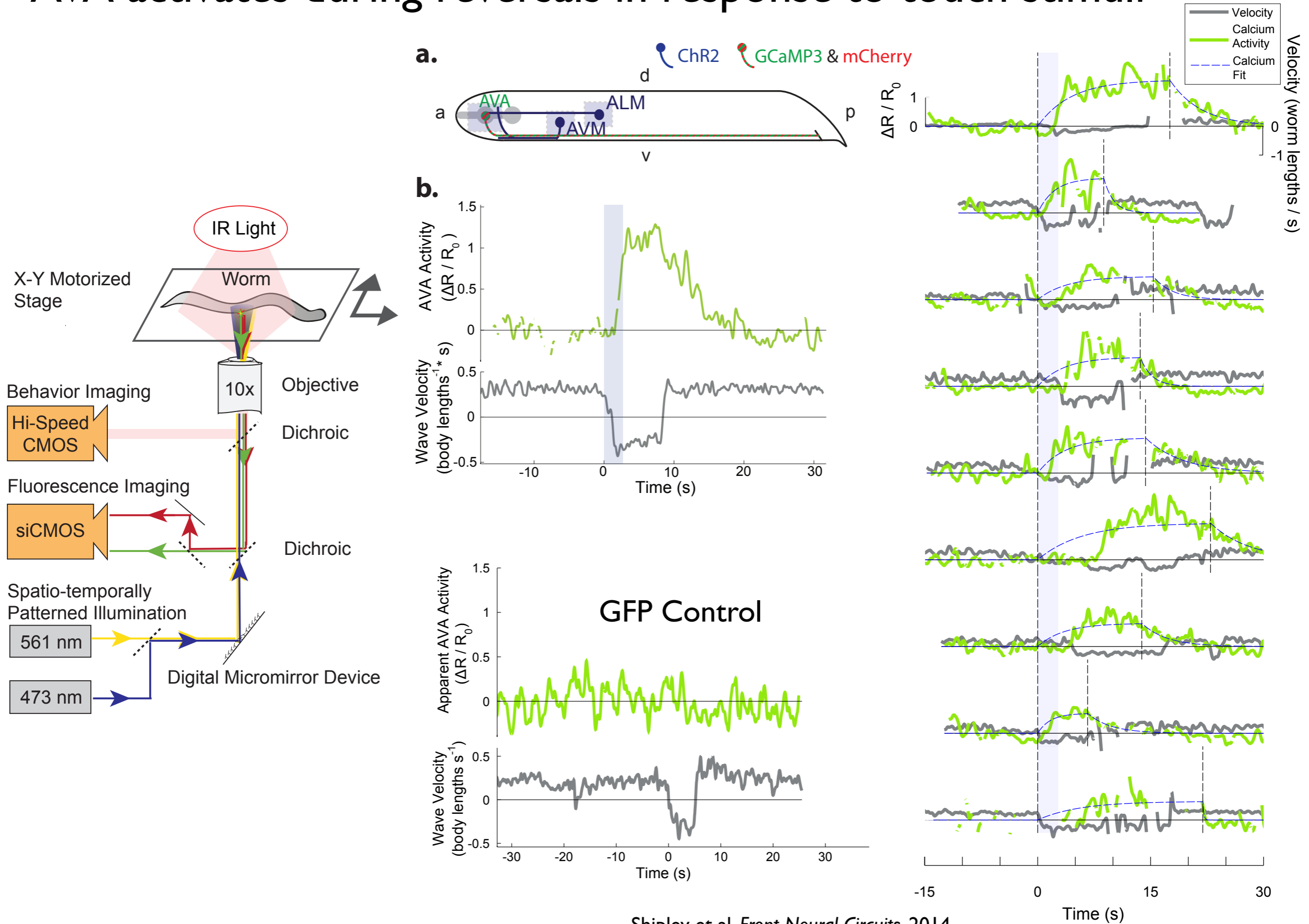


# AVA activates during reversals in response to touch stimuli



Shiple et al, *Front Neural Circuits*, 2014

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Shipley et al, *Front Neural Circuits*, 2014

# Current limitations of calcium imaging & optogenetics in freely moving worms

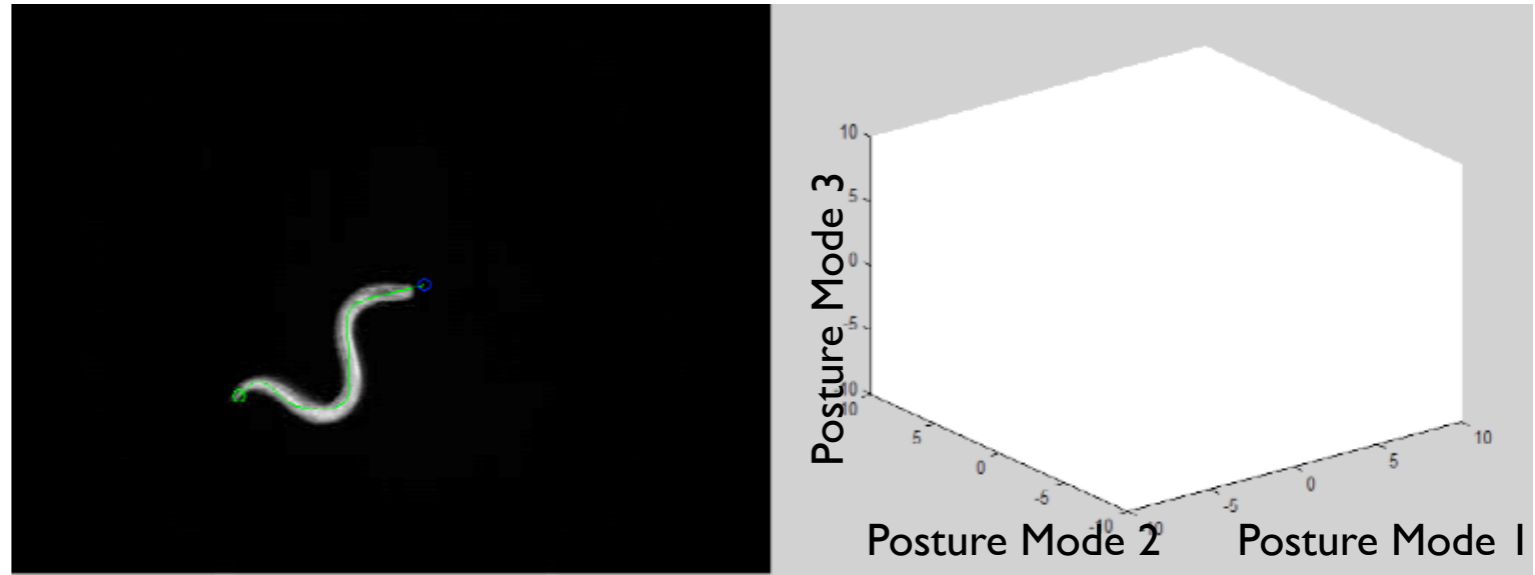
- A few neurons at a time from worms with sparse expression
- No z-sectioning
- Opsins and indicators must be on separate promoters and separate cells
- Simple descriptions of behavior



# Future directions

- Expanded optogenetic toolbox (R-GECl; voltage indicators, brighter GCaMPs etc)
- Richer behavioral descriptions (Stevens et al., 2010)
- Bringing 3D imaging (Schroedel et al., 2013) and 3D stimulation to the moving worm

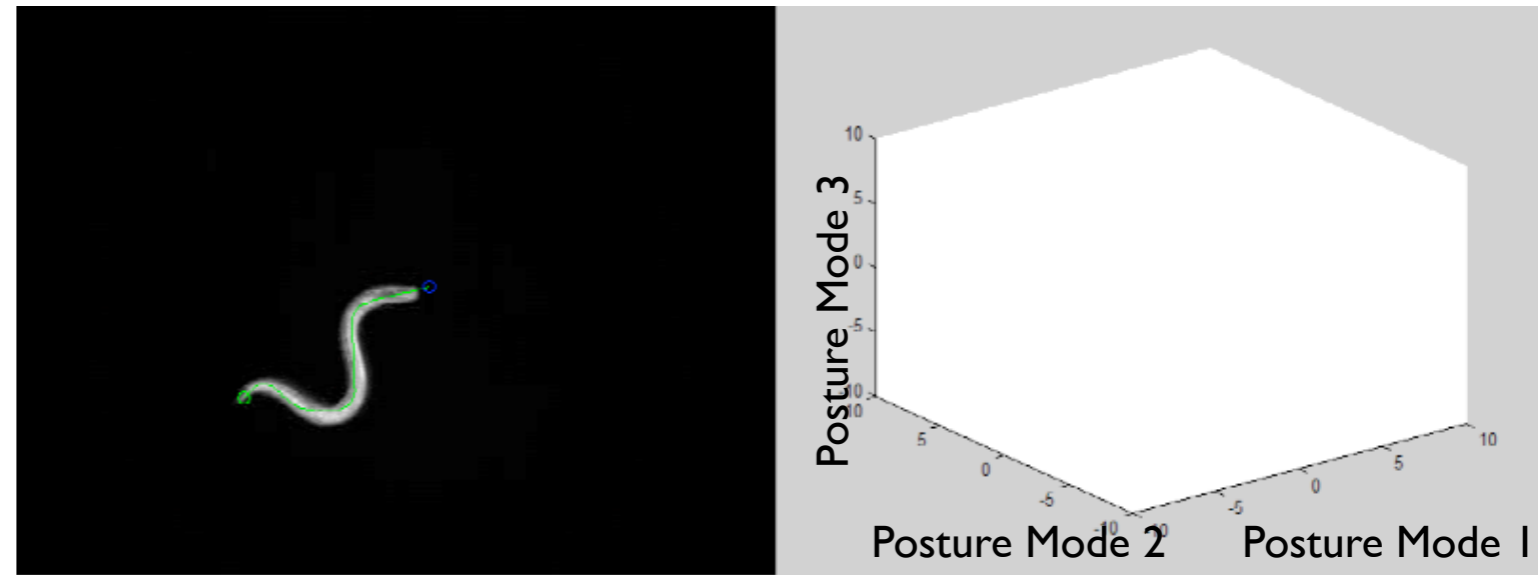
# Future directions



“Eigenworm analysis”

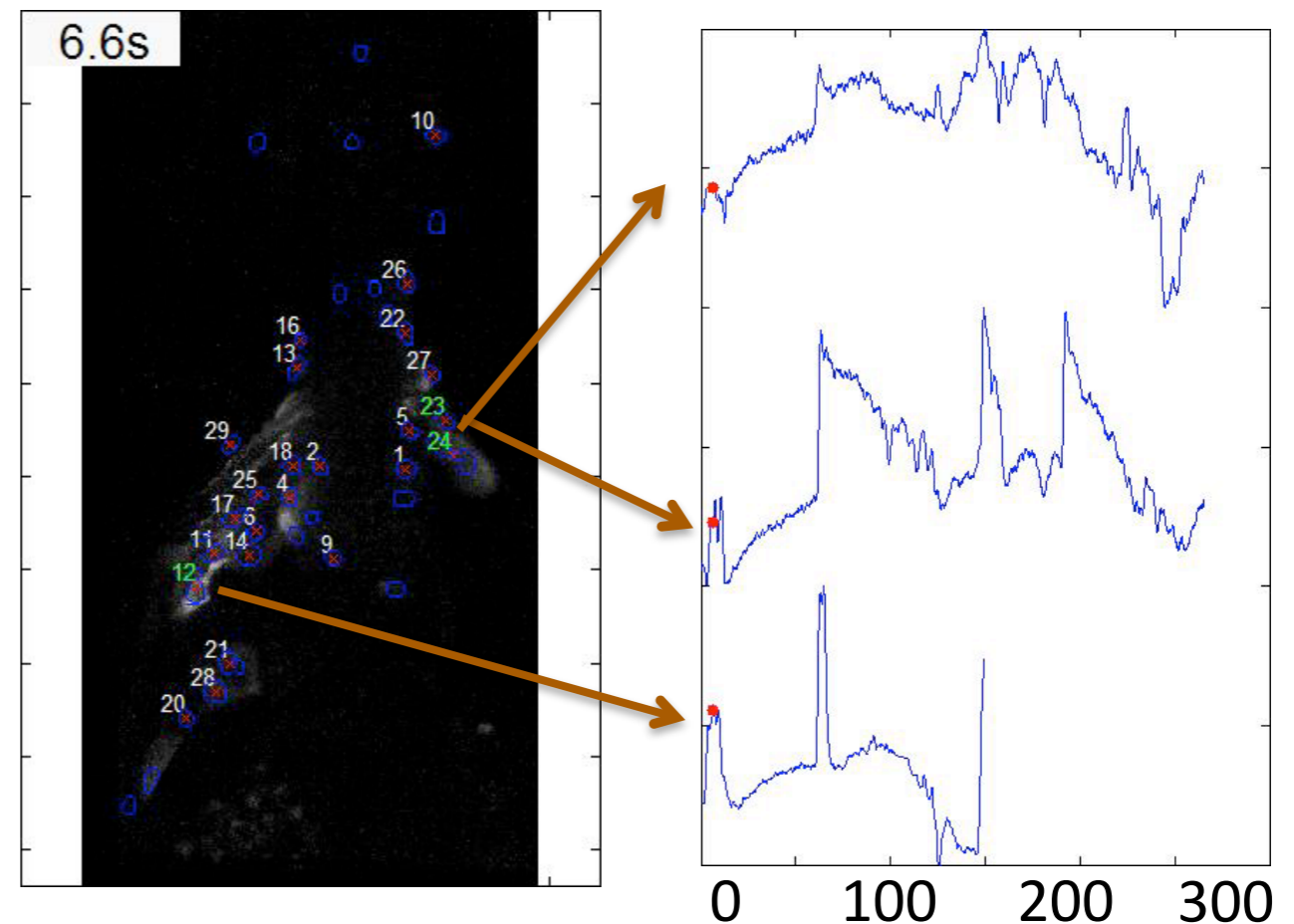
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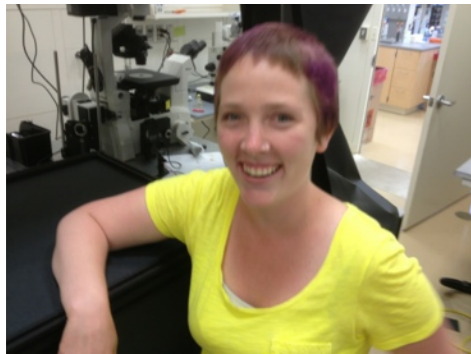
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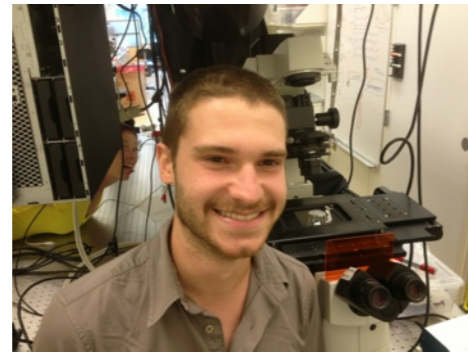
Whole brain imaging in immobilized worms

# The Leifer Lab

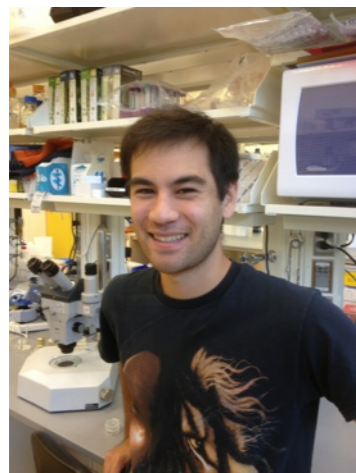
# Collaborators



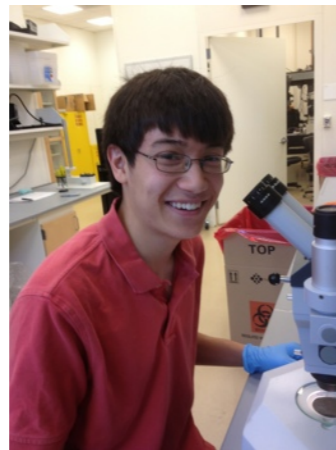
Ashley Linder



Fred Shipley



George Plummer



Kevin Mizes



Chris Clark  
Alkema Lab  
UMass Worcester

Poster 65



Mark Alkema  
UMass Worcester

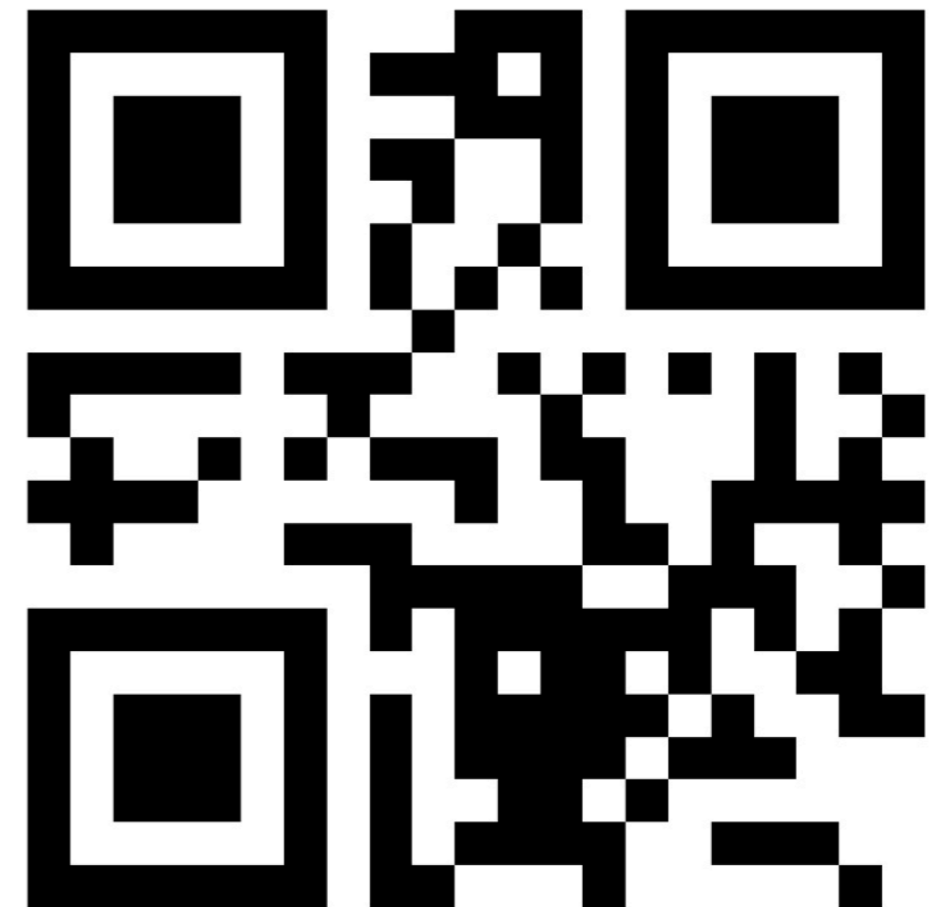


Aravi Samuel  
Harvard



Chris Fang-Yen  
UPenn

## Bibliography:



## Funding:

SIMONS FOUNDATION

Advancing Research in Basic Science and Mathematics

Slides will be posted at [leiferlab.princeton.edu](http://leiferlab.princeton.edu)



<https://www.zotero.org/groups/CeNeuroWorkshop2014>