Technical Note:
Ca^{2+} imaging of unequivocally identified neurons in *C. elegans* worms

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This technical note addresses imaging of NeuroPal/GCaMP6s *C. elegans* worms using the Celesta VBCTGRN Light Engine (Lumencor, Beaverton, OR, USA) in conjunction with the emission filters ET 460/36m, ET 520/40m, ET 585/40m, ET 590/50m and ET 670/50m (Chroma Technologies, Bellow Falls, VT, USA). Autofluorescence in imaging of NeuroPal/GCaMP6s *C. elegans* worms using this microscope hardware is also discussed. We demonstrate that this microscope hardware enables the unequivocal disambiguation (and, thus, identification) of all neurons in NeuroPal/GCaMP6s worms and Ca^{2+} imaging of these neurons, with adequate consideration of autofluorescence in *C. elegans*.

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With a body length of ~0.8 mm, transparent tissue that consists of ~1000 somatic cells, including ~300 neurons organized in a complex nervous system [1-3], and complex behavior that comprises locomotion, learning and memory, chemosensation and mechanosensation [4-7], the soil-dwelling nematode *Caenorhabditis elegans* (*C. elegans*) is a perfect model organism to study relationships between the function of neurons (using imaging of a genetically encoded calcium indicator (GECI); hereafter: Ca^{2+} imaging) and the animal's behavior (reviewed in [8,9]).

A recent breakthrough towards Ca^{2+} imaging of unequivocally identified neurons in *C. elegans* worms is the multi-color, transgene NeuroPAL technology [10,11]. In brief, in NeuroPAL worms, each neuron expresses a specific combination of three landmark fluorophores (mTagBFP2; CyOFP and mNeptune2.5) and a pan-neuronal fluorophore (TagRFP-T). Combining the signal intensities of these fluorophores is then used to identify individual neurons and generate a rich RGB palette shown in NeuroPAL images [10].

All neurons in adult hermaphrodite and male NeuroPAL *C. elegans* worms can be disambiguated, as well as all neurons at all stages of larval development [10]. The NeuroPAL strain is available from the Caenorhabditis Genetics Center at the University of Minnesota (Minneapolis, MN, USA) [12]. For Ca^{2+} imaging, NeuroPAL worms can be combined with expression of the GECI GCaMP6s [10] (hereafter: NeuroPal/GCaMP6s worms).

Instructions how to configure a microscope for imaging NeuroPAL worms have been published [13]. However, these instructions (i) do not show the actual excitation and emission spectra of the fluorophores used in the NeuroPAL technology and of GCaMP6s, (ii) do not describe a device that provides all lasers necessary to perform imaging of unequivocally identified neurons in NeuroPal/GCaMP6s worms in a single, turnkey laser light engine, and (iii) do not address autofluorescence in *C. elegans* that is well known to *C. elegans* microscopists (e.g. [14,15]) and therefore must be considered in imaging of NeuroPal/GCaMP6s worms. This technical note provides the relevant information.

Imaging of NeuroPal/GCaMP6s *C. elegans* worms using the Celesta VBCTGRN Light Engine (Lumencor, Beaverton, OR, USA)

Figure 1 (on Page 3) shows the excitation spectra of the fluorophores used in NeuroPal/GCaMP6s worms, as well as the spectral output provided by the Celesta VBCTGRN Light Engine (Lumencor).

There are two key advantages of using the Celesta VBCTGRN Light Engine (Lumencor) for imaging NeuroPal/GCaMP6s worms: (i) each output is refined by a bandpass filter and merged into a common optical train, passed through a despeckler and directed to a light output port that has a built-in adapter for facile connection to microscopes through a SMA- or FC/ PC-terminated optical fiber (output power at the distal end of the optical fiber is ~800 mW from each laser); and (ii) TTL trigger inputs are provided for all outputs for applications requiring fast (100 µs) switching time.

The relative excitation values obtained by imaging the fluorophores used in NeuroPal/GCaMP6s worms using the Celesta VBCTGRN Light Engine (Lumencor) are summarized in Table 1.

Figure 1 and Table 1 demonstrate that (i) using the wavelengths 405 nm, 488 nm, 518 nm, 545 nm and 577 nm provided by the Celesta VBCTGRN Light Engine (Lumencor) results in full coverage of the fluorophores used in NeuroPal/GCaMP6s worms (values in boldface in Table 1), (ii) that the relative excitation value of each of these fluorophores is at least 70% when using the aforementioned lasers of the Celesta VBCTGRN Light Engine (Lumencor), but (iii) due to overlap in the spectra of the different fluorophores emission filters must be used when imaging NeuroPal/GCaMP6s worms using the Celesta VBCTGRN Light Engine (Lumencor) or any other source of excitation light.

Table 1: relative excitation values obtained by imaging the fluorophores used in NeuroPal/GCaMP6s worms using the Celesta VBCTGRN Light Engine (Lumencor, Beaverton, USA). Values in boldface represent the excitation lasers that are suitable for imaging NeuroPal/GCaMP6s worms. Details (including the explanation of the value in red) are in the text. The relative excitation values were determined using FPbase [16,17] under the CC BY-SA 4.0 license [18].

Fluorophore / Wavelength [nm]	**405**	446	477	**488**	**518**	**545**	**577**	637	680	748
mTagBFP2	**0.96**	0.05	0	0	0	0	0	0	0	0
GCaMP6s	0.02	0.19	0.62	**0.85**	0.15	0	0	0	0	0
CyOFP	0.10	0.47	0.85	0.96	**0.99**	0.54	0.04	0	0	0
TagRFP-T	0.02	0.04	0.08	0.12	0.38	**0.76**	0.24	0	0	0
mNeptune	0.03	0.03	0.05	0.07	0.20	0.43	**0.70**	0.15	0	0

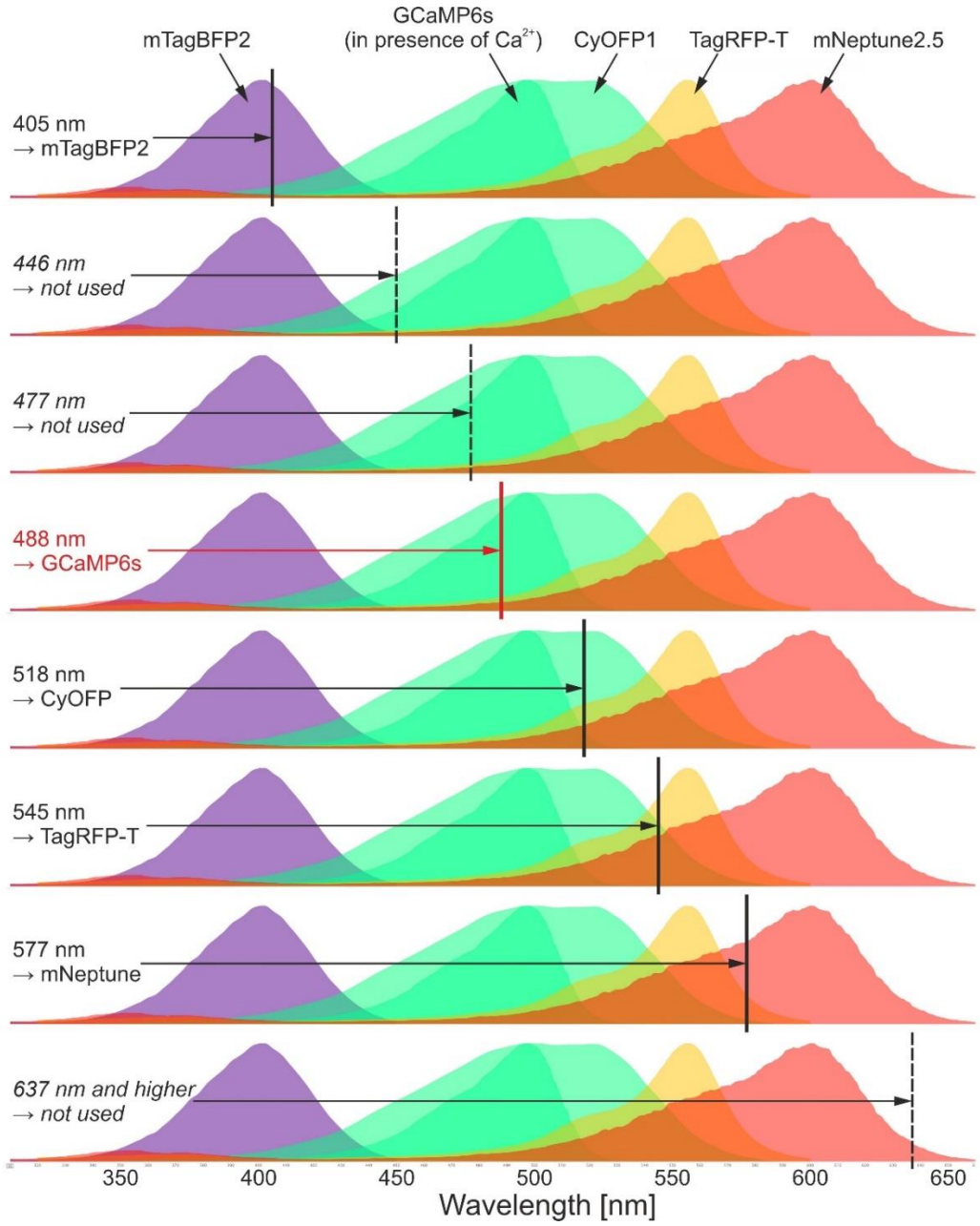

Figure 1: excitation spectra of the fluorophores used in NeuroPal/GCaMP6s worms, as well as the spectral output provided by the Celesta VBCTGRN Light Engine (Lumencor, Beaverton, OR, USA). Details are in the text. The excitation spectra were created using FPbase [16,17] under the CC BY-SA 4.0 license [18].

Imaging NeuroPal/GCaMP6s *C. elegans* worms using the emission filters ET 460/36m, ET 520/40m, ET 585/40m, ET 590/50m and ET 670/50m (Chroma Technologies, Bellow Falls, VT, USA)

Figure 2A (on Page 4) shows the excitation spectra of the fluorophores used in NeuroPal/GCaMP6s worms together with those wavelengths provided by the Celesta VBCTGRN Light Engine (Lumencor) that are suitable for imaging NeuroPal/GCaMP6s worms (c.f. Figure 1 and Table 1), and Figure 2B the emission spectra of the fluorophores used in NeuroPal/GCaMP6s worms. In Figure 2C the bandpass windows of five emission filters (ET 460/36m, ET 520/40m, ET 585/40m, ET 590/50m and ET 670/50m; Chroma Technologies) are overlaid over the emission spectra of the fluorophores used in NeuroPal/GCaMP6s worms.

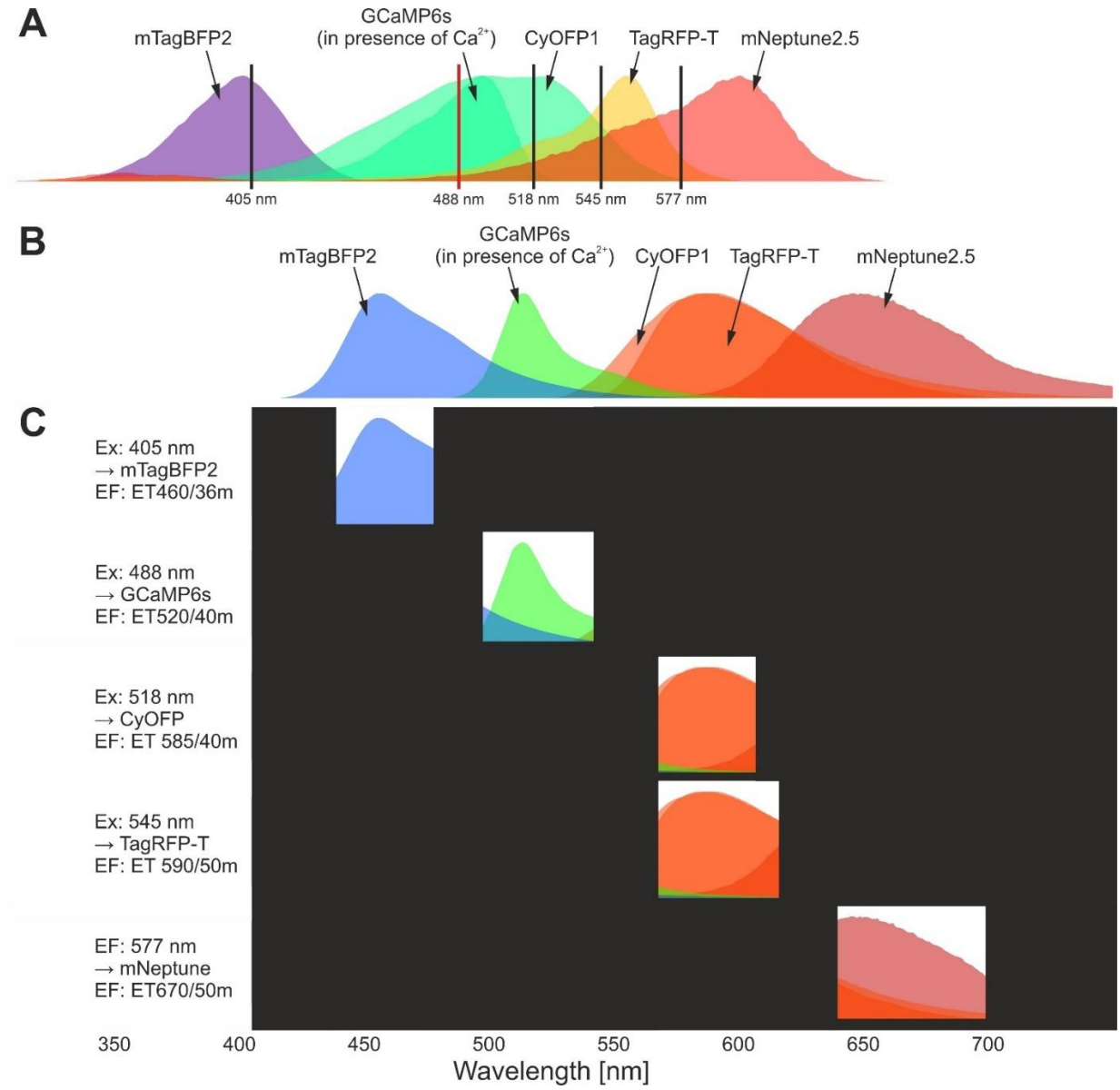

Figure 2: (**A**) excitation spectra of the fluorophores used in NeuroPal/GCaMP6s worms together with those wavelengths provided by the Celesta VBCTGRN Light Engine (Lumencor, Beaverton, OR, USA) that are suitable for imaging NeuroPal/GCaMP6s worms; (**B**) emission spectra of the fluorophores used in NeuroPal/GCaMP6s worms; and (**C**) bandpass windows of five emission filters (ET 460/36m, ET 520/40m, ET 585/40m, ET 590/50m and ET 670/50m; Chroma Technologies, Bellow Falls, VT, USA) overlaid over the emission spectra of the fluorophores used in NeuroPal/GCaMP6s worms. Details are in the text. The excitation and emission spectra were created using FPbase [16,17] under the CC BY-SA 4.0 license [18].

Table 2 summarizes the relative amount of light emitted by each of the fluorophores used in NeuroPal/GCaMP6s worms assuming that the specified excitation laser would excite the corresponding fluorophore by 100% (data were generated by measuring the areas under the curves shown in Figure 2B and 2C using Fiji [19]). However, as shown in Table 1 this assumption is not correct. Therefore, Table 3 summarizes the relative amount of light emitted by each of the fluorophores used in NeuroPal/GCaMP6s worms considering the relative excitation values shown in Table 1.

For example, one may conclude from Figure 3C that the fluorophore TagRFP-T would emit approximately 50% of its maximum light emission when imaging NeuroPal/GCaMP6s worms with the emission filter ET585/40m (value in red in Table 2). However, this would only apply if excitation would be performed using brightfield imaging without excitation filters. In contrast, when using a 518 nm excitation laser the relative amount of

light emitted by the fluorophore TagRFP-T is only 38% of its maximum light emission (red value in Table 1) and, thus, the fluorophore TagRFP-T emits less than 20% of its maximum light emission when imaging NeuroPal/GCaMP6s worms using a 518 nm excitation laser with the emission filter ET585/40m (red value in Table 3).

Figure 2 and Table 3 demonstrate that imaging NeuroPal/GCaMP6s worms using the following combinations of excitation lasers and emission filters (i) Combination 1 {405 nm / ET 460/36m}, Combination 2 {488 nm / ET 520/40m} and Combination 5 {577 nm / ET 670/50ms} results in emission light that is exclusively (Combination 1) or almost exclusively (Combinations 2 and 5) emitted by the fluorophores mTagBFP2 (Combination 1), GCaMP6s (Combination 2) and mNeptune2.5 (Combination 5); (ii) Combination 3 {518 nm / ET 585/40m} results in emission light that is predominantly emitted by the fluorophore CyOPF, and (iii) Combination 4 {545 nm / ET 590/50m} results in emission light that is predominantly emitted by the fluorophores TagRFP-T and CyOFP.

Table 2: relative amount of light emitted by the fluorophores used in NeuroPal/GCaMP6s worms and passed by the specified emission filter (EF) under the assumption that the specified excitation laser (EL) would excite the corresponding fluorophore by 100% (all emission filters from Chroma Technologies, Bellow Falls, VT, USA). Details (including the explanation of the value in red) are in the text. The relative values were determined using FPbase [16,17] under the CC BY-SA 4.0 license [18].

	EL: 405 nm	**EL: 488 nm**	**EL: 518 nm**	**EL: 545 nm**	**EL: 577 nm**
Fluorophore	**EF: ET460/36m**	**EF: ET520/40m**	**EF: ET585/40m**	**EF: ET590/50m**	**EF: ET670/50m**
mTagBFP2	57.8%	0.0%	0.0%	0.0%	0.0%
GCaMP6s	0.0%	81.2%	4.3%	0.0%	0.0%
CyOFP	0.0%	1.6%	48.2%	59.9%	0.0%
TagRFP-T	0.0%	1.7%	48.7%	61.5%	16.4%
mNeptune	0.0%	0.2%	5.7%	10.4%	50.2%

Table 3: relative amount of light emitted by the fluorophores used in NeuroPal/GCaMP6s worms and passed by the specified emission filter (EF), considering the relative excitation values shown in Table 1. Values in boldface represent the combinations of excitation lasers and emission filters that are suitable for imaging NeuroPal/GCaMP6s worms (all emission filters from Chroma Technologies, Bellow Falls, VT, USA). Details (including the explanation of the value in red) are in the text. The relative values were determined using FPbase [16,17] under the CC BY-SA 4.0 license [18].

	EL: 405 nm	**EL: 488 nm**	**EL: 518 nm**	**EL: 545 nm**	**EL: 577 nm**
	EF: ET460/36m	**EF: ET520/40m**	**EF: ET585/40m**	**EF: ET590/50m**	**EF: ET670/50m**
mTagBFP2	**55.5%**	0.0%	0.0%	0.0%	0.0%
GCaMP6s	0.0%	**69.0%**	0.6%	0.0%	0.0%
CyOFP	0.0%	1.6%	**47.7%**	32.3%	0.0%
TagRFP-T	0.0%	0.2%	18.5%	**46.7%**	3.9%
mNeptune	0.0%	0.0%	1.1%	4.5%	**35.6%**

Thus, neurons in NeuroPal/GCaMP6s worms that emit a bright signal when imaging using Combination 3 {518 nm / ET 585/40m} as well as Combination 4 {545 nm / ET 590/50m} are neurons that express CyOFP, whereas neurons that emit a bright signal when imaging using Combination 4 {545 nm / ET 590/50m} but only a weak signal when imaging using Combination 3 {518 nm / ET 585/40m } are neurons that express TagRFP-T.

In summary, the combinations of the lasers with the wavelengths 405 nm, 488 nm, 518 nm, 545 nm and 577 nm provided by the Celesta VBCTGRN Light Engine (Lumencor) and the emission filters ET 460/36m, ET 520/40m, ET 585/40m, ET 590/50m and ET 670/50m (Chroma Technologies, Bellow Falls) enable to unequivocally disambiguate (and, thus, identify) all neurons in NeuroPal/GCaMP6s worms and perform Ca^{2+} imaging of these neurons.

Consideration of autofluorescence in imaging of NeuroPal/GCaMP6s *C. elegans* worms using the Celesta VBCTGRN Light Engine (Lumencor, Beaverton, OR, USA) in conjunction with the emission filters ET 460/36m, ET 520/40m, ET 585/40m, ET 590/50m and ET 670/50m (Chroma Technologies, Bellow Falls, VT, USA)

Figure 3 (taken from [14]) shows spectral properties of autofluorescent materials in *C. elegans* worms as follows: (i) contours containing 75% of the overall fluorescence emission intensity for several common autofluorescent biomolecules (tryptophan (W), collagen IV, NADH, NADPH, riboflavin and FAD) as reported in [20]; (ii) 75% contours of autofluorescent peaks for living and dead *C. elegans* worms based on data from Coburn *et al*. [21]; and (iii) the range analyzed in an earlier study of *C. elegans* autofluorescence by Gerstbrein *et al*. [22].

According to Pincus *et al*. [14] the intestine and the uterus are the two main sources of autofluorescence in *C. elegans* worms, and autofluorescence appears to be due to a spatially heterogeneous mix of substances with distinct spectral properties. Furthermore, blue autofluorescence in *C. elegans* worms remains static until a peak near death, red autofluorescence increases almost linearly with time, and green autofluorescence (primarily found in the intestine) seems to combine both trends [14].

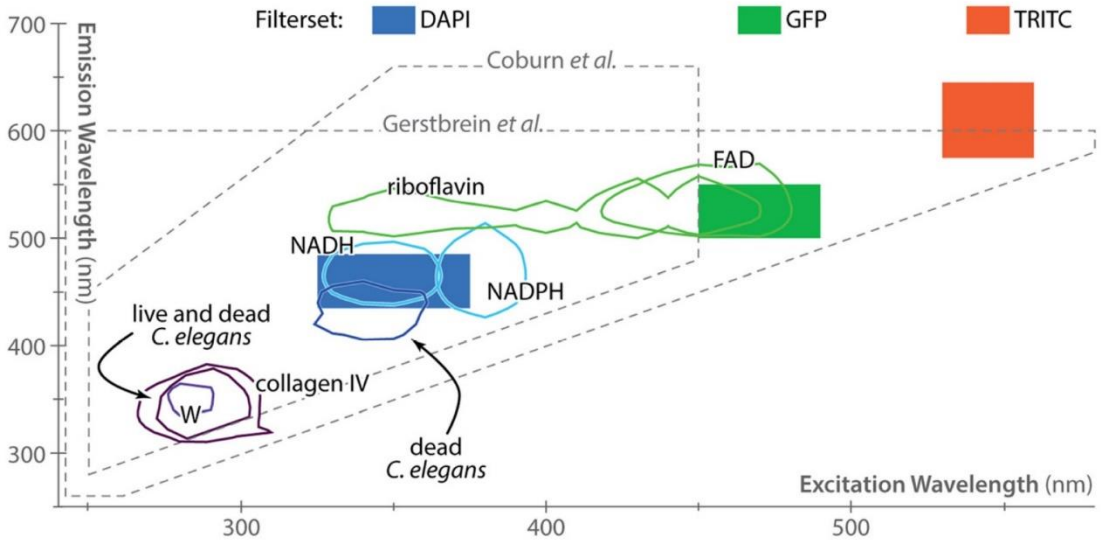

Figure 3: spectral properties of autofluorescent materials in *C. elegans* worms. Details are in the text. The figure was taken from [14] under the CC BY 3.0 license [23].

Figure 4A shows Figure 3 together with spectral properties of imaging transgenic LSD2022 *C. elegans* worms using a brightfield fluorescence microscope and a commonly used single band filter set (Chroma 49002 - ET - EGFP (FITC/Cy2; Chroma Technology) [15]. The LSD2022 *C. elegans* worms express an integrated collagen::GFP transgene (ROL-6::GFP) which is visible in the cuticula (white arrow in Figure 4B and Figure 5B) as well as GFP driven by the *ttx-3* promoter in the AIY interneuron pair (white arrowhead in Figure 4B and Figure 5B) [24]. The corresponding spectra of excitation light (vertical black arrow in Figure 4A) and emission light (horizontal black arrow and box indicated by the red arrow in Figure 4A) are indicated. As shown in Figure 4B (taken from [15]) this combination of excitation light, excitation filter and emission filter did not enable to distinguish between the GFP signal and *C. elegans* autofluorescence.

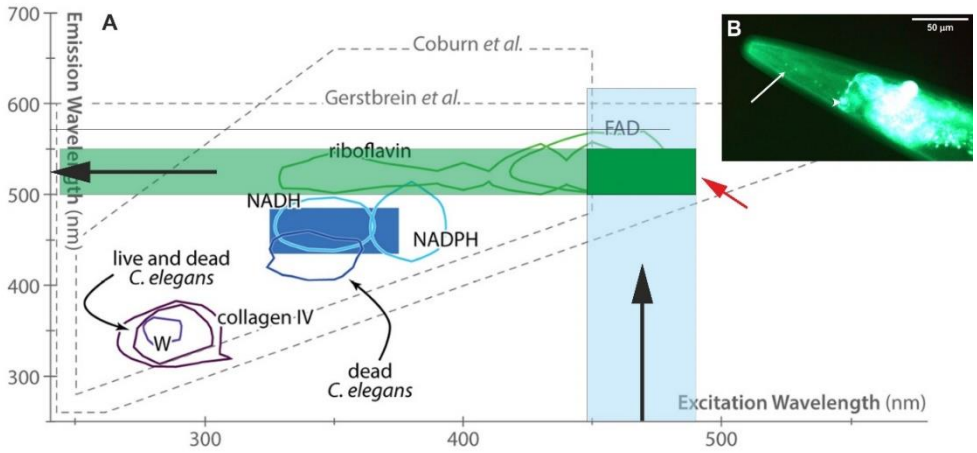

Figure 4: (**A**) spectral properties of autofluorescent materials in *C. elegans* worms, overlaid with spectral properties of imaging transgenic LSD2022 *C. elegans* worms using a brightfield fluorescence microscope and a commonly used single band filter set (Chroma 49002 - ET - EGFP (FITC/Cy2; Chroma Technology) [15]; and (**B**) photomicrograph of the head of a LSD2022 *C. elegans* worm captured using the microscope hardware described in (A). Details are in the text. Panel A (without the overlays) was taken from [14] under the CC BY 3.0 license [23]; Panel B was taken from [15] with permission from the authors as well as from Bio-protocol LLC (Sunnyvale, CA, USA).

Figure 5A (on Page 8) shows Figure 3 together with spectral properties of imaging transgenic LSD2022 *C. elegans* worms using a brightfield fluorescence microscope, an ET 485/10x excitation filter (Chroma Technology) and a 69000 ET-DAPI/FITC/TRITC (69000x, 69000m, 69000bs) excitation/emission filter (Chroma Technology) from which the 69000x excitation filter was removed [15]. The corresponding spectra of excitation light (vertical black arrow) and emission light (horizontal black arrows and boxes indicated by red arrows) are indicated in Figure 5. As shown in Figure 5B (taken from [15]) this combination of excitation light, excitation filter and emission filters enabled to distinguish between the GFP signal and *C. elegans* autofluorescence.

Figure 6 shows Figure 3 together with spectral properties of three methods of imaging *C. elegans* worms:

- Method 1: imaging transgenic LSD2022 *C. elegans* worms as shown in Figure 4 (green horizontal box indicated by a black asterisk in Figure 6);
- Method 2: imaging transgenic LSD2022 *C. elegans* worms as shown in Figure 5 (boxes indicated by red arrows in Figure 6); and
- Method 3: imaging NeuroPal/GCaMP6s worms using the Celesta VBCTGRN Light Engine (Lumencor) in conjunction with the emission filters ET 460/36m, ET 520/40m, ET 585/40m, ET 590/50m and ET 670/50m (Chroma Technologies) (c.f. Figure 3C and Table 3) (vertical lines and boxes indicated by black arrows).

Figure 6 demonstrates the following: (i) all wavelengths used in Method 3 are outside the 75% overall fluorescence emission intensities reported in [20] for the common autofluorescent biomolecules tryptophan, collagen IV, NADH, NADPH, riboflavin and FAD; (ii) except for 405 nm all wavelengths used in Method 3 are outside the 75% contours of autofluorescent peaks for living and dead *C. elegans* worms reported in the aforementioned study by Coburn *et al.* [21]; (iii) the wavelength 577 nm used in Method 3 is outside the range of C. *elegans* autofluorescence analyzed in the aforementioned study by Gerstbrein *et al*. [22]; and (iv) the range of wavelengths emitted by GCaMP6s in Method 3 ("1" in Figure 6) is only slightly larger than the range of wavelengths emitted by GFP in Method 2 ("2" in Figure 6) but smaller than the range of wavelengths emitted by GFP in Method 1 ("3" in Figure 6).

In summary, imaging NeuroPal/GCaMP6s *C. elegans* worms using the Celesta VBCTGRN Light Engine (Lumencor, Beaverton, OR, USA) in conjunction with the emission filters ET 460/36m, ET 520/40m, ET 585/40m, ET 590/50m and ET 670/50m (Chroma Technologies, Bellow Falls, VT, USA) does adequately consider autofluorescence in *C. elegans*.

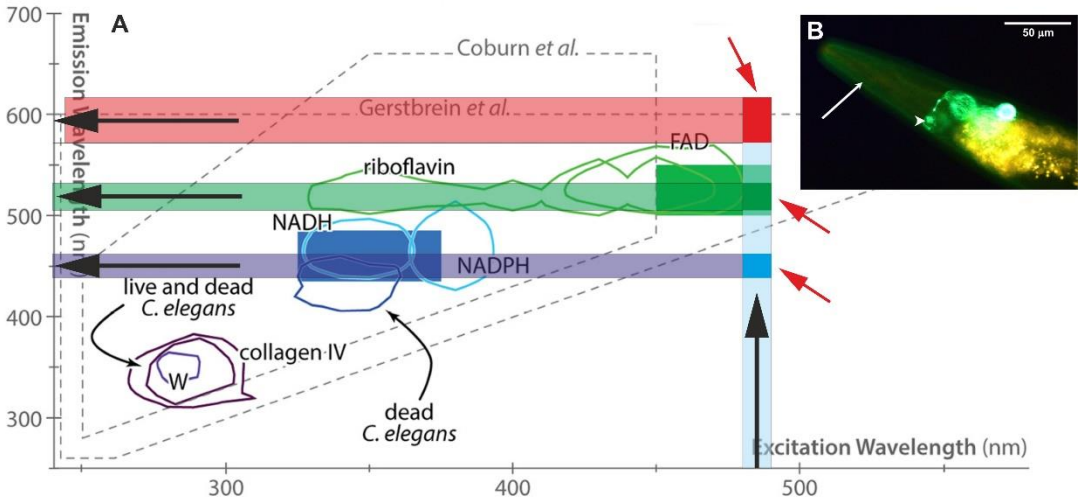

Figure 5: (**A**) spectral properties of autofluorescent materials in *C. elegans* worms, overlaid with spectral properties of imaging transgenic LSD2022 *C. elegans* worms using a brightfield fluorescence microscope, an ET 485/10x excitation filter (Chroma Technology) and a 69000 ET-DAPI/FITC/TRITC (69000x, 69000m, 69000bs) excitation/emission filter (Chroma Technology) from which the 69000x excitation filter was removed [15]; and (**B**) photomicrograph of the head of a LSD2022 *C. elegans* worm captured using the microscope hardware described in (A). Details are in the text. Panel A (without the overlays) was taken from [14] under the CC BY 3.0 license [23]; Panel B was taken from [15] with permission from the authors as well as from Bio-protocol LLC (Sunnyvale, CA, USA).

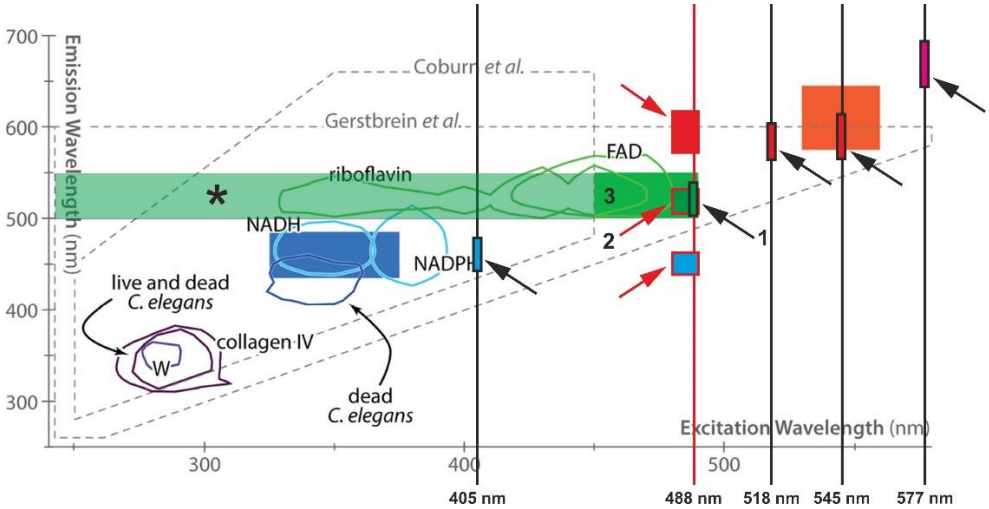

Figure 6: spectral properties of autofluorescent materials in *C. elegans* worms, overlaid with spectral properties of three methods of imaging *C. elegans* worms: Method 1: imaging transgenic LSD2022 *C. elegans* worms as shown in Figure 4 (green horizontal box indicated by a black asterisk); Method 2: imaging transgenic LSD2022 *C. elegans* worms as shown in Figure 5 (boxes indicated by red arrows); and Method 3: imaging NeuroPal/GCaMP6s worms using the Celesta VBCTGRN Light Engine (Lumencor) in conjunction with the emission filters ET 460/36m, ET 520/40m, ET 585/40m, ET 590/50m and ET 670/50m (Chroma Technologies) (c.f. Figure 3C and Table 3) (vertical lines and boxes indicated by black arrows). Details are in the text. The figure (without the overlays) was taken from [14] under the CC BY 3.0 license [23].

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