

GFP Magnetofluorescence

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This project is maintained by [Maria Ingaramo](#) in the [York lab](#), and was funded by [Calico Life Sciences LLC](#)

Appendix

Note that this is a limited PDF or print version; animated and interactive figures are disabled. For the full version of this article, please visit https://andrewgyork.github.io/gfp_magnetofluorescence

Magnetic control of GFP-like fluorescent proteins

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Raw data

The raw data used to generate the main text figures is available at doi.org/10.5281/zenodo.8136927 and doi.org/10.5281/zenodo.8137093

Instrumentation

We used a Leica DMI8 microscope with a 10x objective and a SpectraX light source to deliver 1.7 ms of 1.1 W/cm² power per camera exposure through a 470/24 nm excitation filter, or 1.4 W/cm² per camera exposure through a 550/15 nm excitation filter every 30 ms. We used the same quad dichroic (Leica) for all experiments and either a 540/21 nm

emission filter to image green proteins (like EGFP) or a 705/72 nm filter to image red proteins (like mScarlet). In a typical experiment, we move a 1 cm³ N42 neodymium magnet adjacent to/distant from the sample every 5 seconds using a servo motor (HS-422) while acquiring 1.7 ms camera exposures every 30 ms, continuously on a PCO edge 4.2 camera with 4x4 pixel binning. The magnet moved relatively quickly ($\ll 1$ s) controlled by an Arduino UNO, and modulated the magnetic field at the sample between ~0 to ~10 mT (or ~25 mT for *E. coli* samples). The magnet movement and the acquisition were not synchronized, but we started the traces when the magnet was close to the sample.

Sample preparation

Fluorescent protein coated beads: To attach fluorescent proteins to Ni-NTA-coated beads, we first transformed T7 Express *E. coli* (NEB) with pRSET plasmids that encoded fluorescent proteins with an N-terminal 6xHIS tag and selected on LB/Carbenicillin plates. Because we frequently encounter problems with plasmid loss, we inoculate 1 L of LB/Carbenicillin with all of the transformants and grow the liquid culture for ~3 hours at 37°C. After overnight induction with 200 μ M IPTG, samples were pelleted, resuspended, and lysed in 50 mM phosphate buffer pH 8 + 500 mM NaCl with BugBuster. After sample centrifugation, the lysate was incubated with 1 mL of cOmplete Ni-NTA agarose beads (Roche) for 1 hour. The beads were washed 3 times, and stored in 1 mL of 50 mM phosphate buffer pH 8 + 500 mM NaCl. For imaging, we diluted 0.5 μ L of this sample into 100 μ L PBS (Corning 21-040-cv) in a 384-well glass bottom plate (Cellvis). Flavinated-EGFP beads were made similarly, except that we co-transformed *E. coli* with [pRSET-FP-FlavinTag](#) and a pD881-MR vector (ATUM) encoding the flavin transferase ApbE ([pRham-StrepTag-ApbE](#)). Growth was carried out with the addition of Carbenicillin/Kanamycin and 50 μ M riboflavin, and we induced first with 0.2% rhamnose overnight and then with 200 μ M IPTG for 6 hours the next day. To purify mScarlet with a Streptag instead of a 6xHIS, we used streptactin superflow plus beads (Qiagen) instead of Ni-NTA beads. For imaging beads, we used an additional 1.6x magnification lens on the emission path.

Metabolite screen: We screened a library of 730 [metabolites](#) [[Hicks 2023](#)] on our microscope, using protein-coated Ni-NTA beads and a 1x mag changer, a quad dichroic, a 470/24 nm excitation filter, and a 540/21 nm emission filter for GFP, and 550/15 nm and 705/72 nm for mScarlet. We also screened both proteins using the corresponding

emission filters, with 405, 470, 560 and 640 nm concomitant illumination, but did not get any additional magnetoresponsive hits. The metabolites' final concentration was 700 μM in PBS.

Pure fluorescent protein in solution samples: Purified protein was provided by Wuxi Biortus. 4 μL samples (with final concentrations of 5.1 μM mScarlet or 19 μM EGFP in PBS) were covered with 3 μL mineral oil and imaged in 1536-well glass bottom plates. We used 1536-well glass bottom plates because the field-of-view (FOV) covers the whole well, so diffusion at the FOV-edges is less of a concern. Concentrations were determined using extinction coefficients of 55900 $\text{M}^{-1}\text{cm}^{-1}$ at 488 nm for EGFP, 100000 $\text{M}^{-1}\text{cm}^{-1}$ at 569 nm for mScarlet, and 11300 $\text{M}^{-1}\text{cm}^{-1}$ at 450 nm for FAD and FMN. For the other metabolites, we dissolved the powder in either water or DMSO to make 50 mM stocks, depending on their solubility.

Mammalian cell experiments: We transfected Cos7 cells with [pCDH plasmids](#) expressing mito-EGFP-FlavinTag and a flavin transferase separated by a T2A sequence in a glass bottom 8-well chamber (Cellvis). Samples were imaged ~48 hours after transfection, using a 40x objective, a 470/24 nm excitation filter, and a 515/30 nm emission filter. The DMEM + 10% FBS (with added glutamax and antibiotics) was replaced with warm imaging solution (Invitrogen A14291DJ) without any additives right before acquisition. The whole microscope was warmed up to 37°C before the start of the experiment, but the incubator turned off while imaging to avoid vibrations. Samples were imaged without supplying CO_2 within an hour of media exchange.

We also transfected EGFP-FlavinTag without a flavin transferase, as well as EGFP without a FlavinTag, both in the cytosol and mitochondria. The magnetic field effect on the fluorescence was ambiguous in these samples. We did not perform any optimizations like adding exogenous flavin to the media, establishing the localization of the ApbE enzyme, or adding a gene for overexpression of a flavin synthase [[Tong 2022](#)]. We suspect that optimization of these parameters is quite important; just because we didn't see magnetoresponse in these samples doesn't mean it wasn't there.

Experimental design

All the experimental results presented in the main text, unless stated, are performed with protein immobilized on Ni-NTA beads. These experiments are performed in 100 μL of the relevant cofactor/PBS solution in a 384-well plate (Cellvis), and the FOV is a small fraction of the whole volume. Experiments with purified protein in solution are performed in 1536-well plates, which have wells that fit entirely within the FOV. All *in vitro* experiments use ~ 0 to ~ 10 mT magnetic field changes. However, *E. coli* samples allowed us to get the magnet closer, so the magnetic field changes between ~ 0 and ~ 25 mT. If no concentration is specified, it means that the experiments contain 5.1 μM mScarlet, 19.1 μM EGFP, 1000 μM FAD or 500 μM FMN in PBS. We carried out experiments at room temperature, except those using mammalian cells, which were at 37°C .

We would like to address some of the experimental pitfalls that we encountered while looking for magnetic field effects on fluorescence. First, reflection from the magnet produced a very fast and large change in signal that can be mistaken for a magnetic field effect, but this can be avoided by covering the sample with black matte paper. Second, we used an electromagnet to corroborate that we can qualitatively obtain similar traces. This indicates that vibrations from the servo movement or heating from the electromagnet are not likely explanations for the observed behavior. Third, we used a long working distance (~ 1.1 cm) air objective to avoid contact with the sample, and reduce the magnet's ability to pull on the objective. The traces are similar whether we use hardware autofocus or not.

We'd also like to highlight a few observations/tips for those interested in repeating our results. Start with a 3 to 5 minute timelapse at the maximum camera framerate and high power, while changing the magnetic field at 5 second intervals. The effects are small and are easy to miss without a repeating pattern to guide the eye. More cofactor is not necessarily better, so testing a few concentrations might be useful. I'd suggest starting with WST-8 (MedChemExpress, HY-D0831), which we dissolved in water to make a 50 mM stock that can be stored frozen at -80°C for at least 6 months. WST-8 works with both EGFP and mScarlet, and unlike flavins, it does not require pre-illumination. To look at mScarlet with flavins, it might be easier to start with 470/560 nm concomitant excitation. It took us a bit of time to get good at observing the modulation, and you might not succeed on the first try. If so, please reach out.

Magnetic field response of fluorescent proteins in *E. coli*

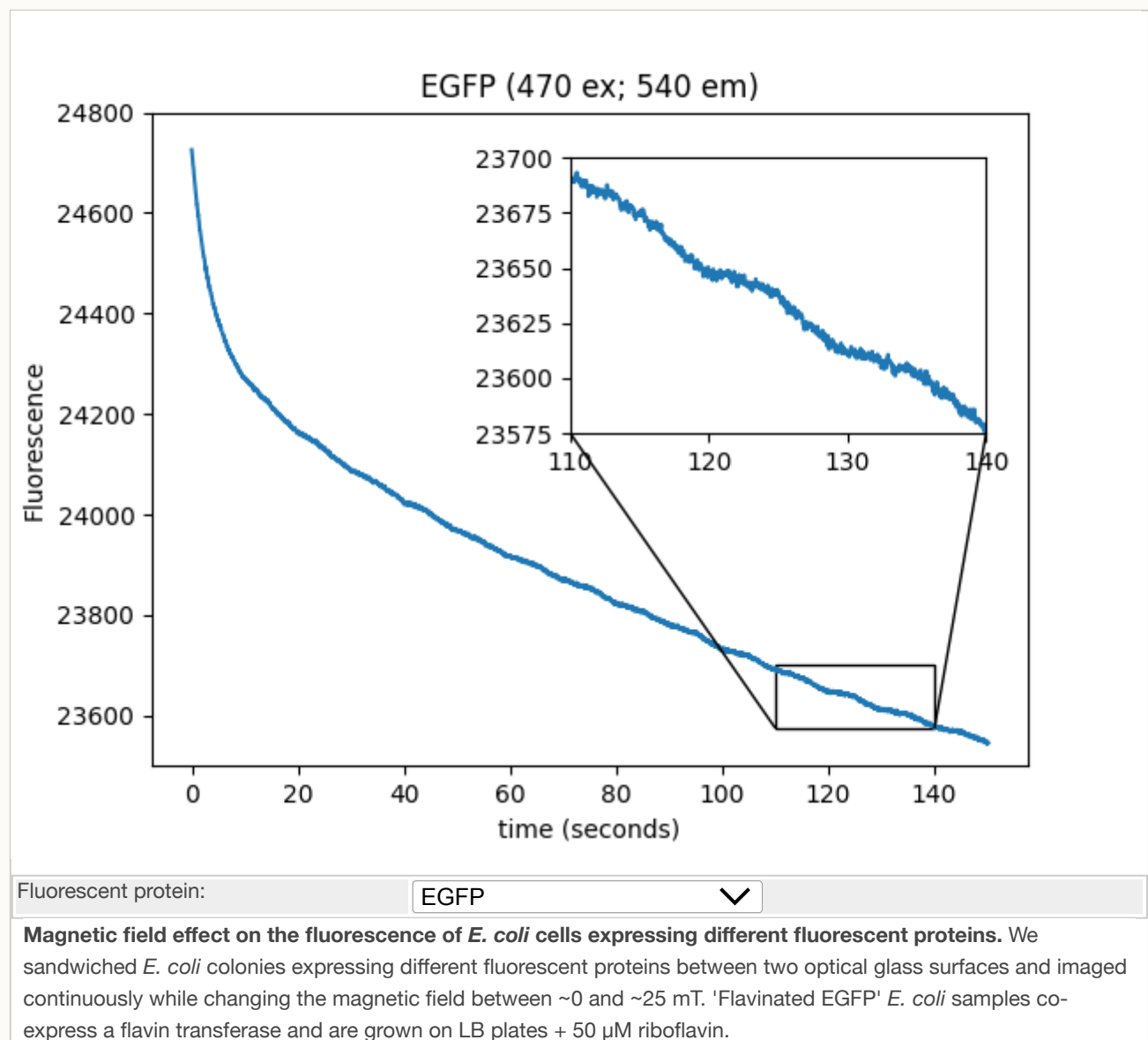
We screened by looking at T7 Express (NEB) cells expressing fluorescent proteins from a pRSET plasmid. A colony was scraped off the LB/carbenicillin plate two days after transformation and sandwiched between a coverslip and a multiwell plate optical lid (Cellvis) turned upside-down. We applied different amounts of pressure to each sample in order to maintain maximum power while avoiding saturating the camera. We believe that high excitation power is important, because some cofactors, like FMN, seem to require production of a photo-induced intermediate. Since these samples are very flat, we could get the magnet closer to the FOV, which produced a magnetic field of ~25 mT. For the most part, we looked at proteins that we already had on hand, except for dmCry W394F [Zollitsch 2018], an FAD binding protein with a mutation that reportedly increased transient absorption spectra magnetoresponse. Among the proteins that we looked at were mCardinal, mCherry, KillerRed, dsRED, mScarlet, pHuji, mVenus, tdVenus, EGFP, StayGold, mCerulean, CyOFP, LSSmOrange2, mMaple, AsLOV V416T C450A, dmCry W394F. AsLOV V416T (residues 404-546) [Kawano 2013] is a mutant that we already had, to which we (on a hunch) added the C450A mutation [Kay 2003], which (luckily!) seemed to increase the magnetoresponse.

In the green/yellow family, we noticed magnetoresponse from mVenus, tdVenus, EGFP, AsLOV V416T C450A, and dmCry W394F. From the red protein family, we noticed magnetoresponse from mScarlet and dsRed. Interestingly, mScarlet did not require 470 nm excitation which suggests that the endogenous cofactor(s) in *E. coli* aren't flavins. Other proteins did not show a convincing response, but we do not rule out that a modulation might be observed under more favorable conditions (like lower background, higher endogenous cofactor levels, better matched imaging wavelengths, etc).

We tested tdVenus-coated beads in PBS, which were not magnetoresponsive, but adding FAD restored weak magnetoresponse. However, FAD *and* ascorbic acid gave a much stronger magnetoresponse [Kattinig 2016]. Because the reaction was very sensitive to relative concentrations of the cofactors, we decided to pursue characterization of EGFP instead, which displayed a strong magnetoresponse without needing ascorbic acid.

We would also like to note that co-expressing a flavin transferase enzyme and adding 50 μ M riboflavin dramatically improves the magnetoresponse of EGFP-FlavinTag in *E. coli*

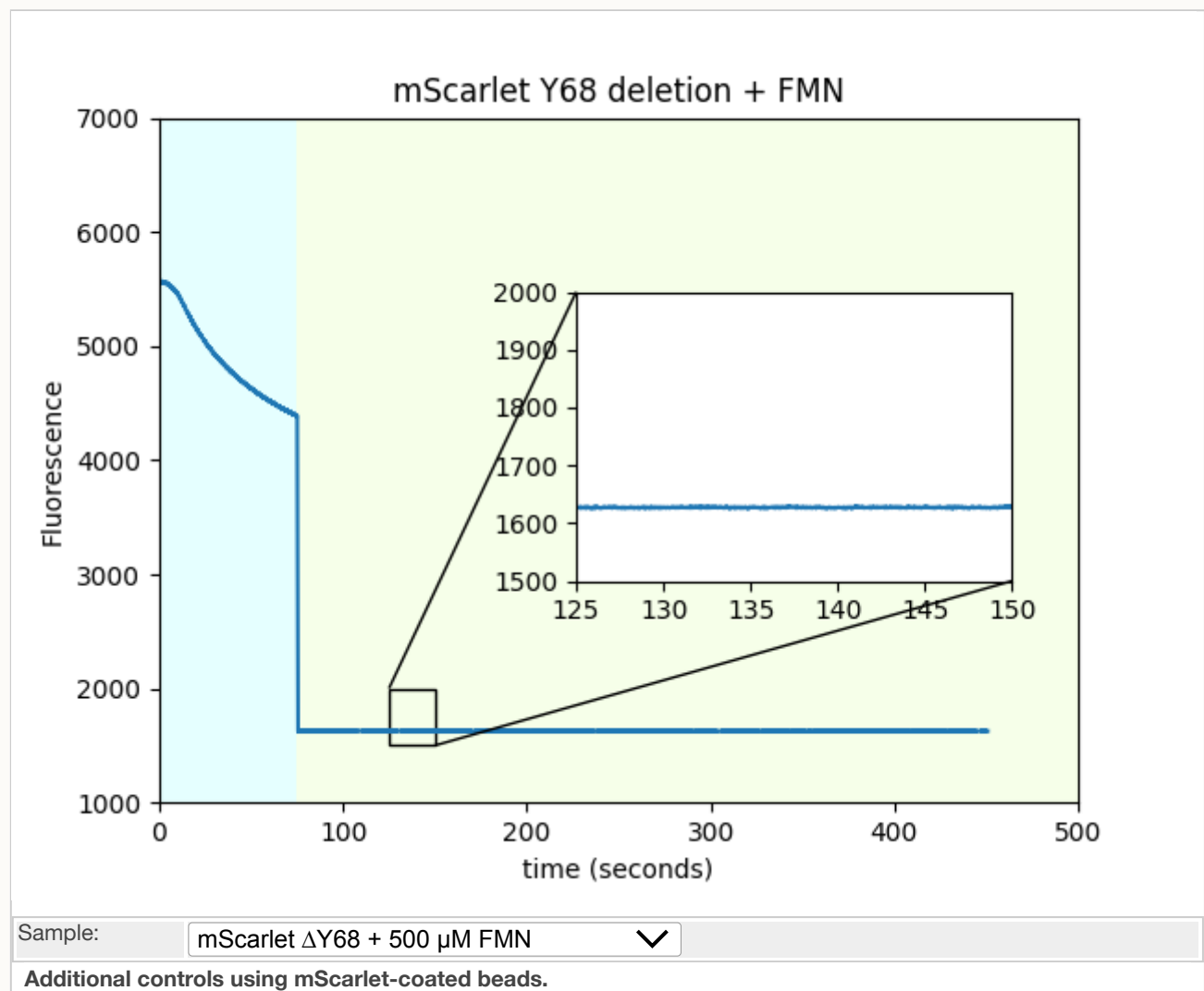
(Figure 1, EGFP-FlavinTag selected).



Additional controls using mScarlet immobilized on beads

We performed several controls to try to establish that FMN and mScarlet are the source of the magnetic field change in fluorescence. First, to rule out that the nickel metal from the Ni-NTA beads is somehow involved in the magnetic field response, we switched the 6xHIS tag for a StrepTag and the Ni-NTA beads for streptactin-coated beads, and observed similar results. Second, in order to establish that mScarlet is indeed the source of the fluorescent magnetoresponse and it is not merely the flavin accumulating on the bead surface, we deleted tyrosine 68 from mScarlet; as shown below, this eliminates magnetoresponse in the red channel. Deletion of the chromophore tyrosine extinguishes the fluorescence while maintaining the overall protein fold, (at least with mVenus, Steven

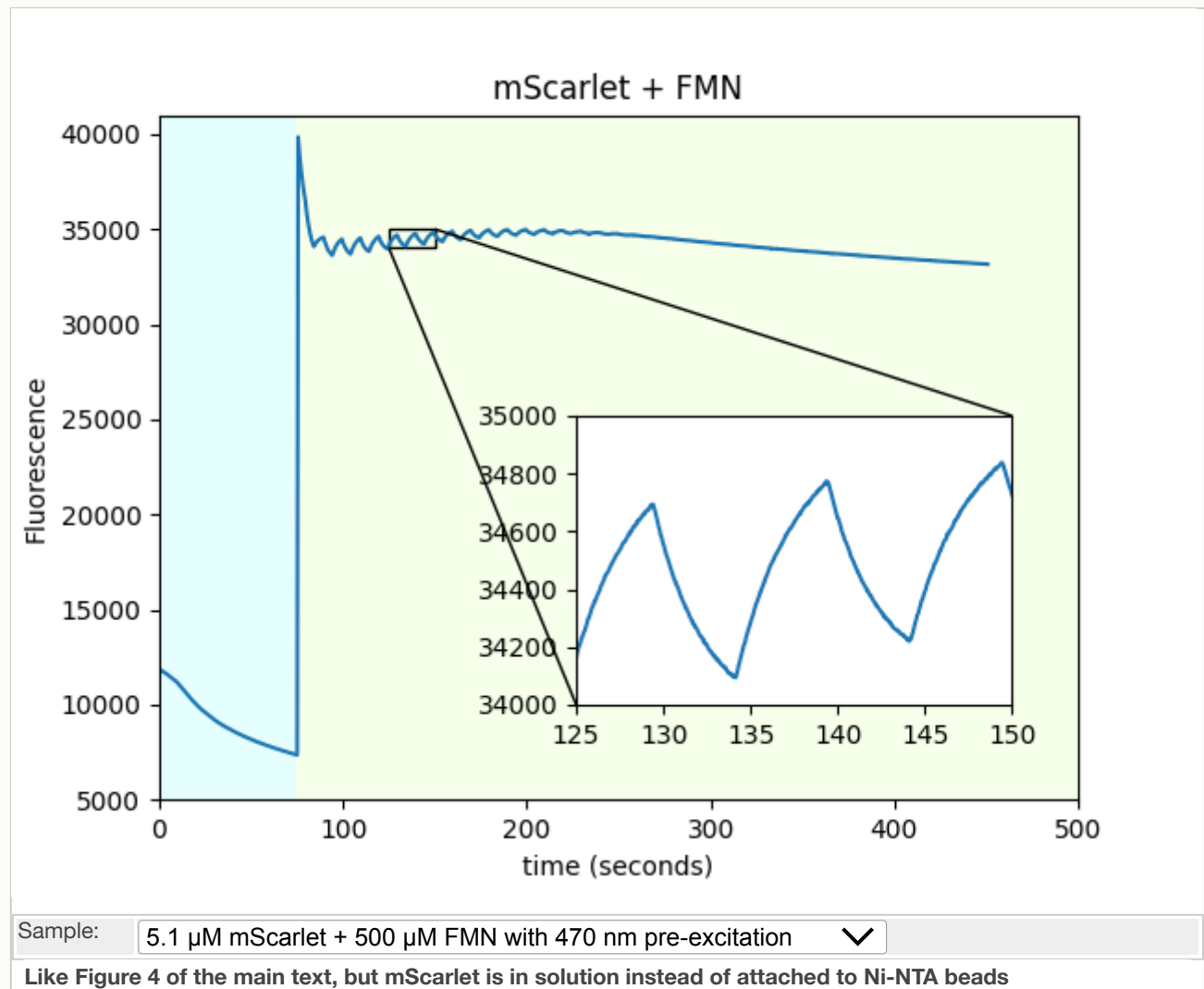
Vogel, personal communication), and we believe it has the same effect on mScarlet. This experiment also suggests that it is unlikely that we are just observing changes in fluorescence originating only from the cofactor abstracting electrons from the fluorescent protein surface without involvement of the fluorescent protein's chromophore. Also in support of the previous point, we used the FlavinTag to covalently attach a flavin to the C-terminus of mScarlet and also mScarlet $\Delta Y68$ and observed no magnetoresponse. Even though we did not quantify the percent flavination, these beads are visibly yellow, indicating that a substantial fraction of the protein has a flavin attached. Finally, and quite interestingly, mScarlet/FAD mixtures do not exhibit magnetoresponse. A possible explanation is that mScarlet binds FMN, but not FAD.



Additional controls using purified mScarlet in solution

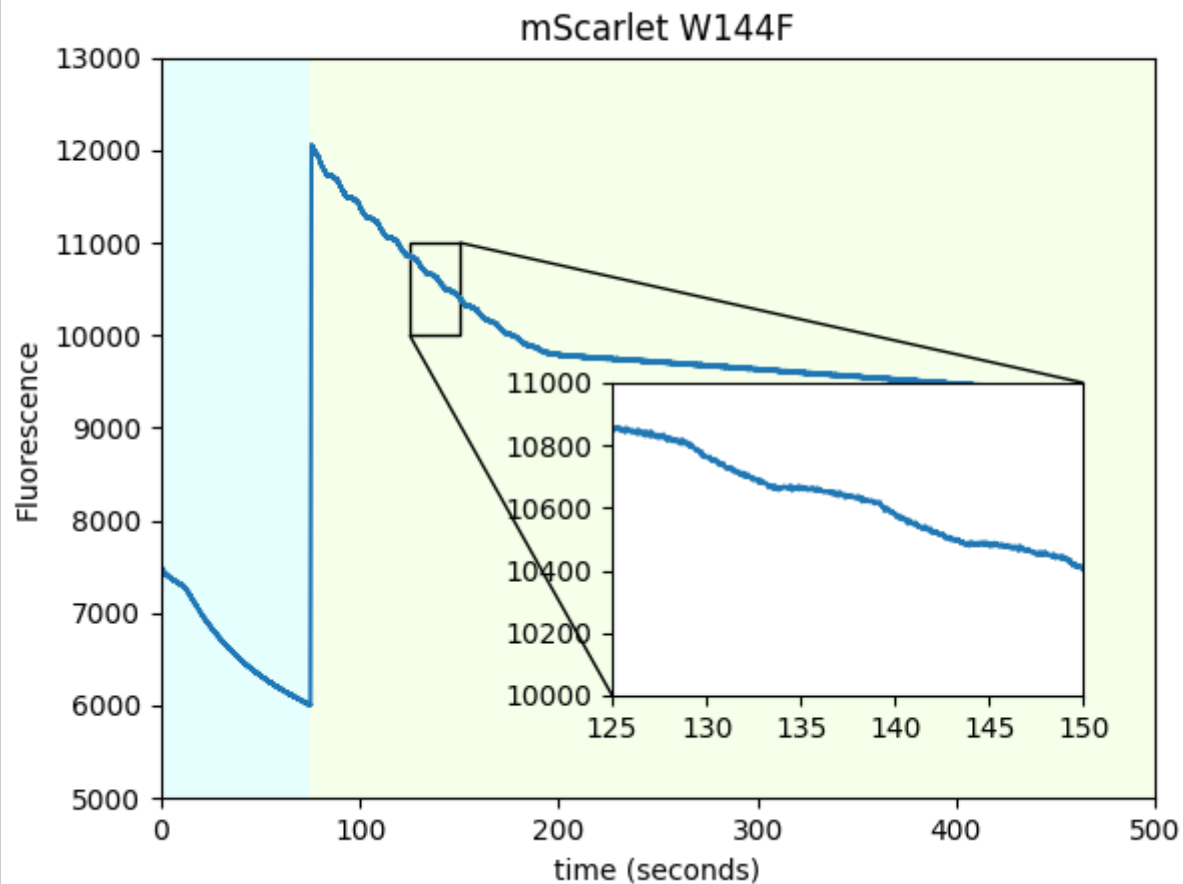
We repeated the experiments from [Figure 4](#) of the main text (which immobilized mScarlet on beads) using pure protein in solution (PBS), in wells small enough to fit in the FOV. This

prevents diffusion artifacts from complicating the interpretation of the traces. We observed similar results qualitatively, indicating that the beads are irrelevant to the modulation of fluorescence by the magnetic field, although quantitatively, the amplitude of the response is lower.



mScarlet tryptophan mutants on beads

We mutated each of the three tryptophans present in mScarlet to probe the hypothesis that one of them would provide a path for electron transfer from the chromophore to the surface of the protein. We found that W144 was the only one that changed the magnetoresponse. However, this mutant produces significantly dimmer beads. We're hesitant to overinterpret this result, but since we have the data, we might as well share it.



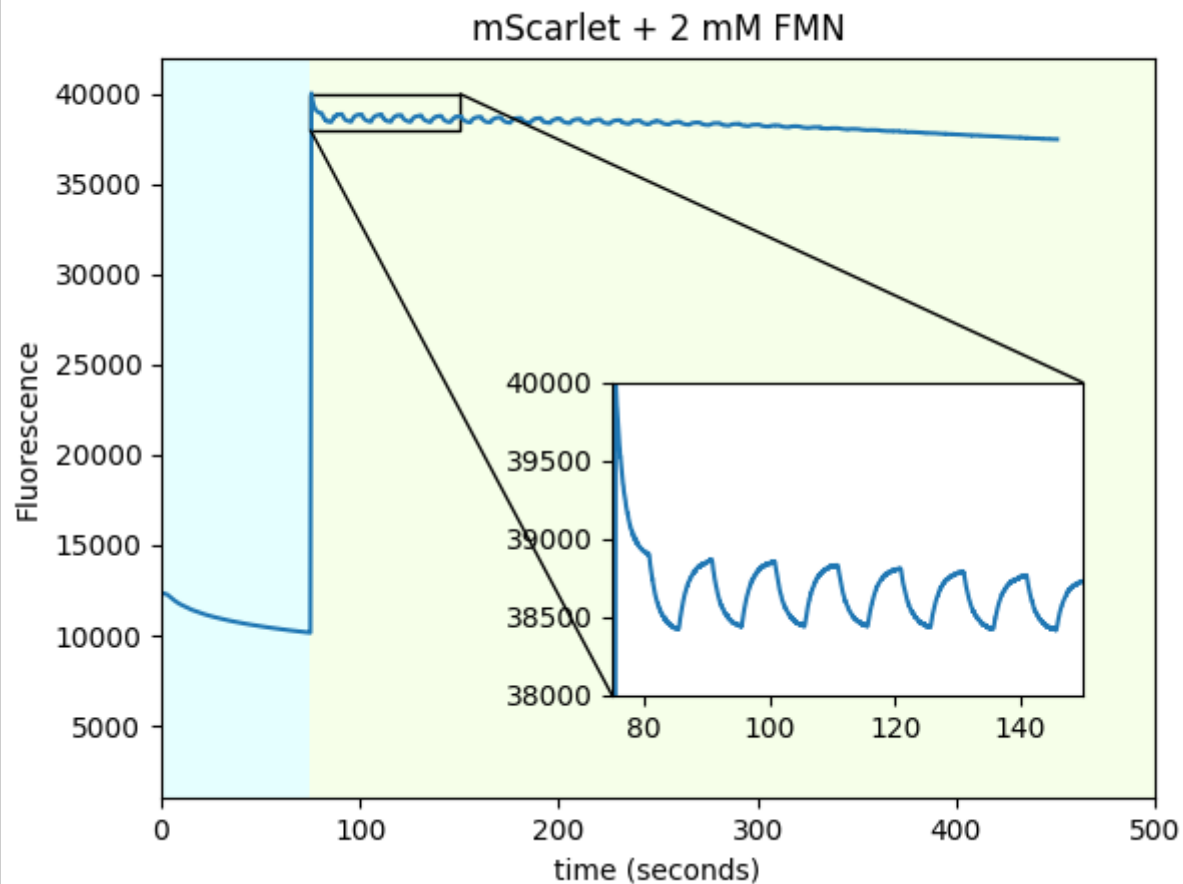
mScarlet tryptophan mutant:

W144F ▼

mScarlet tryptophan mutants. We imaged mScarlet tryptophan single mutant coated beads in the presence of 500 μ M FMN. Tryptophan 144 was the only position that affected the magnetic field response, making it less pronounced and shorter.

Dependence of mScarlet magnetofluorescence on FMN concentration

We used purified mScarlet in solution to determine the fluorescent magnetoresponse as a function of FMN concentration.



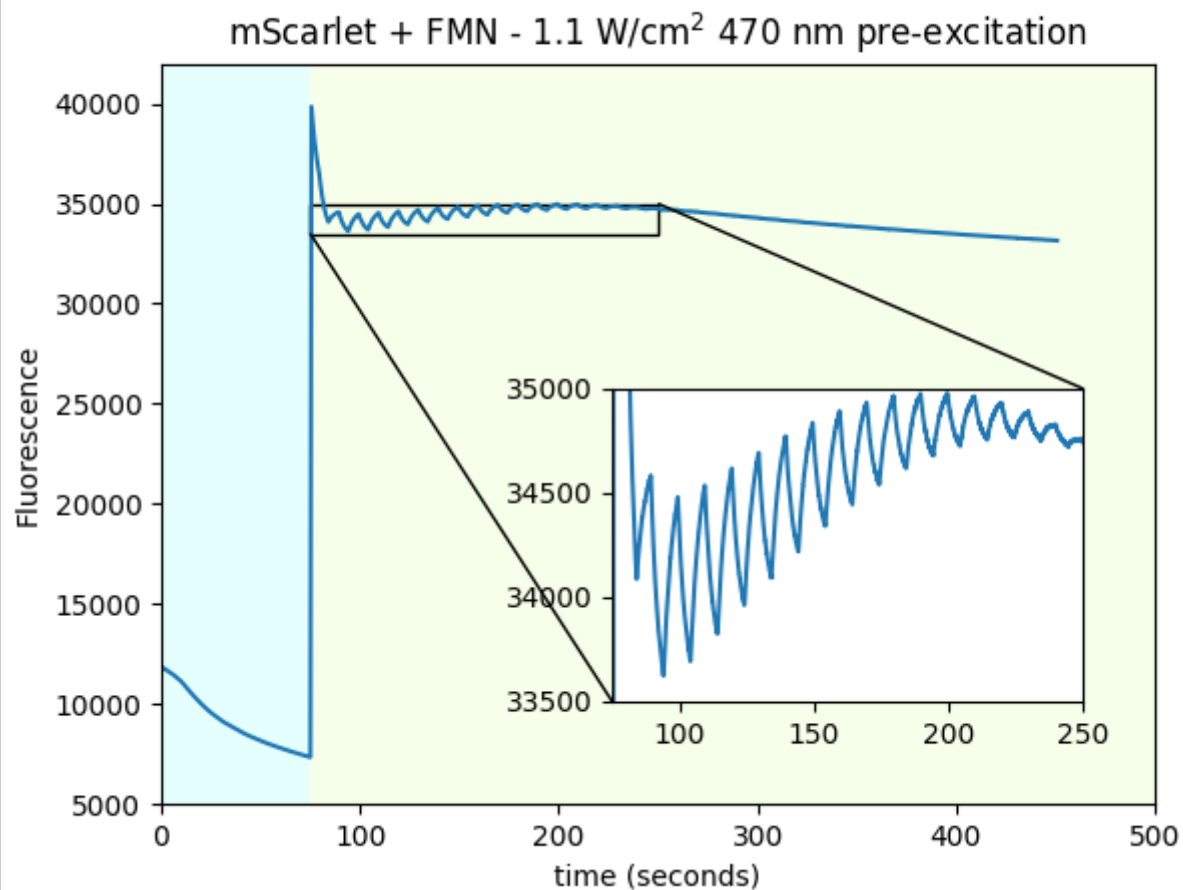
FMN concentration:

2000 μ M ▼

The magnetic field response of mScarlet fluorescence depends on FMN concentration. We imaged 5.1 μ M purified mScarlet protein in PBS in the presence of increasing concentrations of FMN. The whole well fit in an FOV, allowing us to ignore diffusion.

Dependence of mScarlet magnetofluorescence on 470 nm power

We used purified mScarlet in solution to determine the fluorescent magnetoresponse as a function of 470 nm pre-irradiation power.



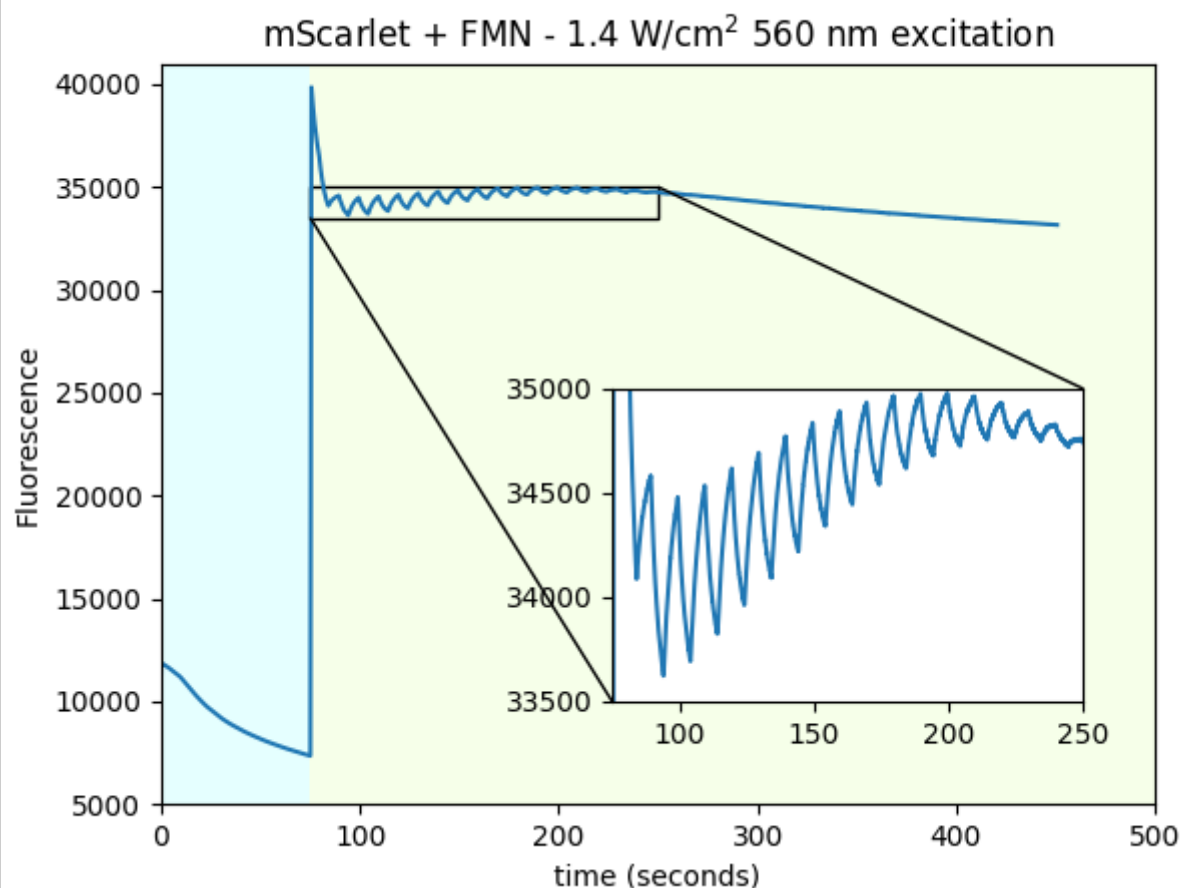
470 nm pre-irradiation power:

1.1 W/cm² ▼

The magnetic field response of mScarlet fluorescence increases with the power of the 470 nm pre-irradiation step. We imaged 5.1 μ M purified mScarlet protein + 500 μ M M FMN in PBS in wells that fit in a single FOV in order to ignore diffusion. The power of the 550 nm LED used to image after the pre-irradiation step was held constant.

Dependence of mScarlet magnetofluorescence on 550 nm power

We used purified mScarlet in solution to determine the fluorescent magnetoresponse as a function of 550 nm imaging power. We observed little dependence, other than absolute intensity, and photobleaching rates.



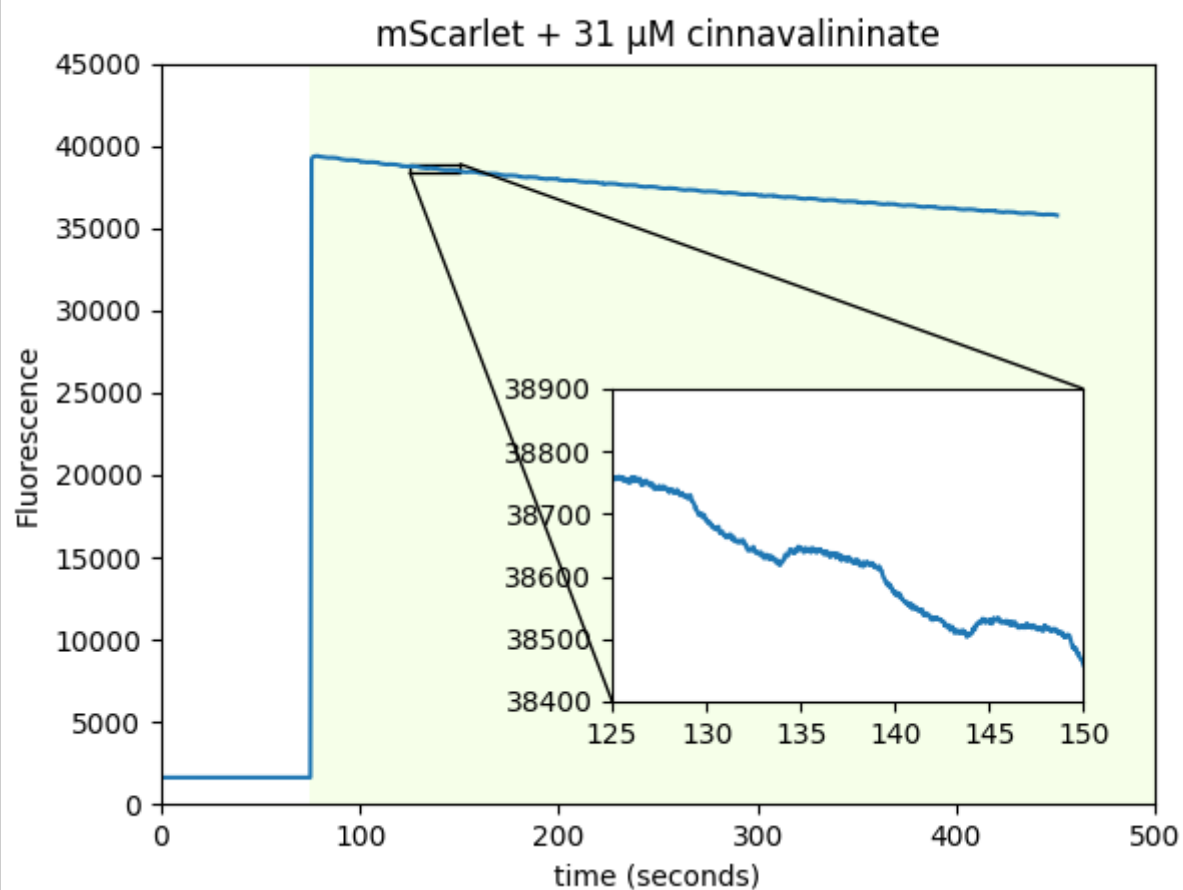
550 nm imaging power

1.4 W/cm² ▼

550 nm imaging power over the range of 0.19 to 1.4 W/cm² has little effect on the duration or rate of the magnetic field response of mScarlet. We imaged 5.1 μ M purified mScarlet protein + 500 μ M FMN in PBS in wells that fit in a single FOV in order to ignore diffusion. The pre-irradiation 470 nm power was the same in all samples.

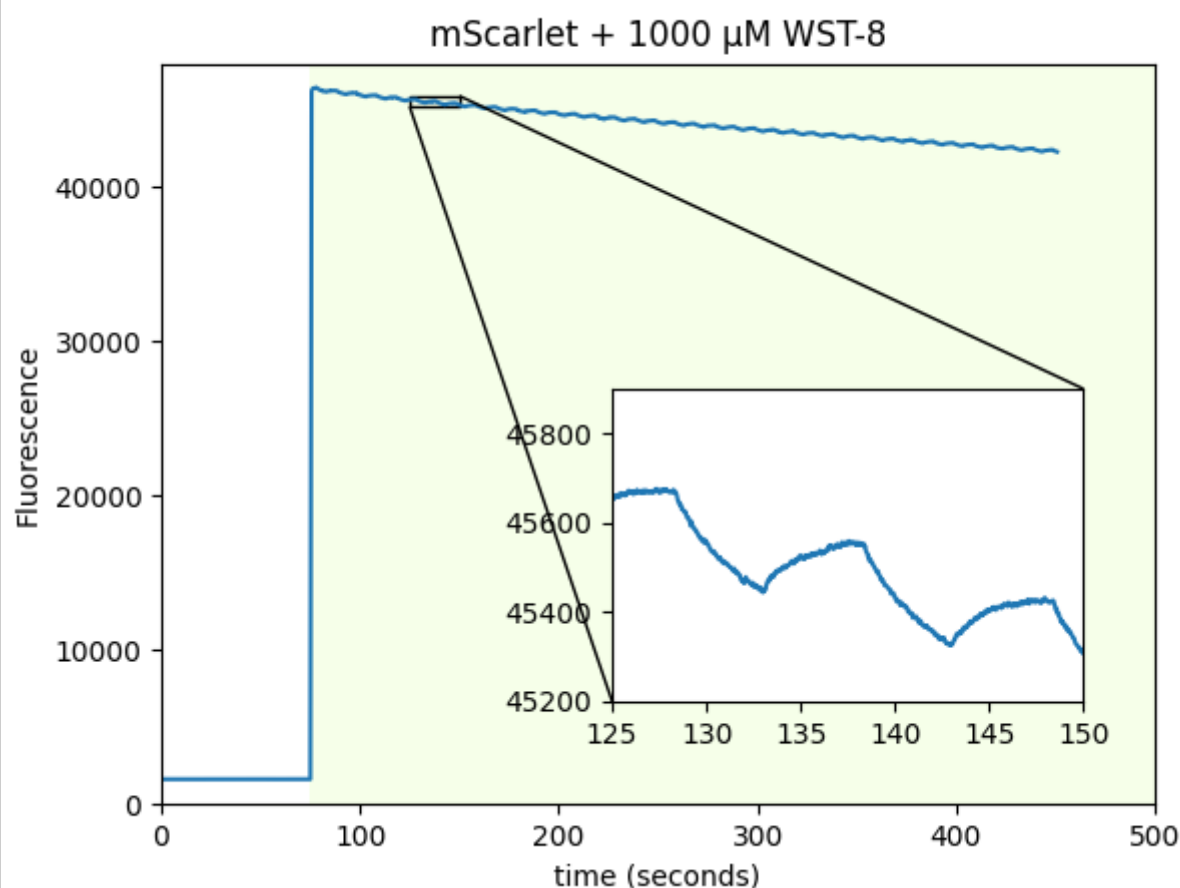
mScarlet magnetofluorescence with non-flavin cofactors

At the suggestion of Adam Cohen, we looked for mScarlet magnetofluorescence with WST-8 as a cofactor instead of FMN. WST-8 is a tetrazolium dye used to assess cellular metabolic activity that forms a 460 nm absorbing formazan dye upon reduction. It works, without needing 470 nm pre-excitation! We also used purified beads coated with mScarlet to screen a library of ~730 [metabolites](#). We screened using 550 nm excitation and a 700 nm emission filter on two 384-well plates. We identified cinnavalininate, an intermediate in tryptophan metabolism, as an additional cofactor. We also ran the screen with 405/470/550/640 nm excitation, and recovered FMN as a positive hit.



Sample: 5.1 μ M mScarlet + 31 μ M cinnavalininate ✓

Magnetic field response of mScarlet/cinnavalininate solutions. We imaged 5.1 μ M mScarlet with varying concentrations of cinnavalininate in 1536 wells. These mixtures did not require 470 nm pre-illumination.



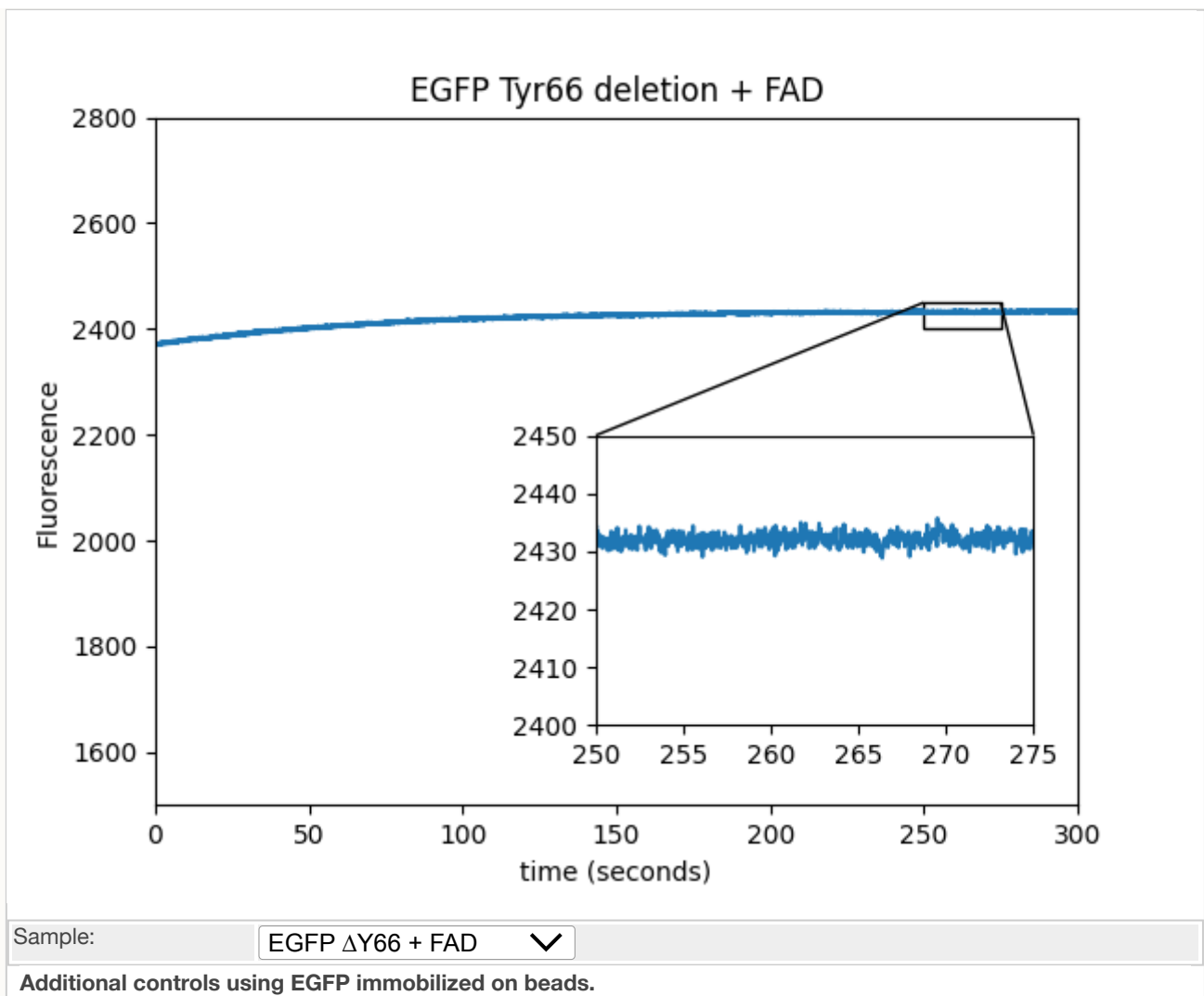
Sample: 5.1 μ M mScarlet + 1000 μ M WST-8 ▼

Magnetic field response of mScarlet/WST-8 solutions. We imaged 5.1 μ M mScarlet with varying concentrations of WST-8 in 1536 wells. These mixtures did not require 470 nm pre-illumination.

Additional controls using EGFP immobilized on beads

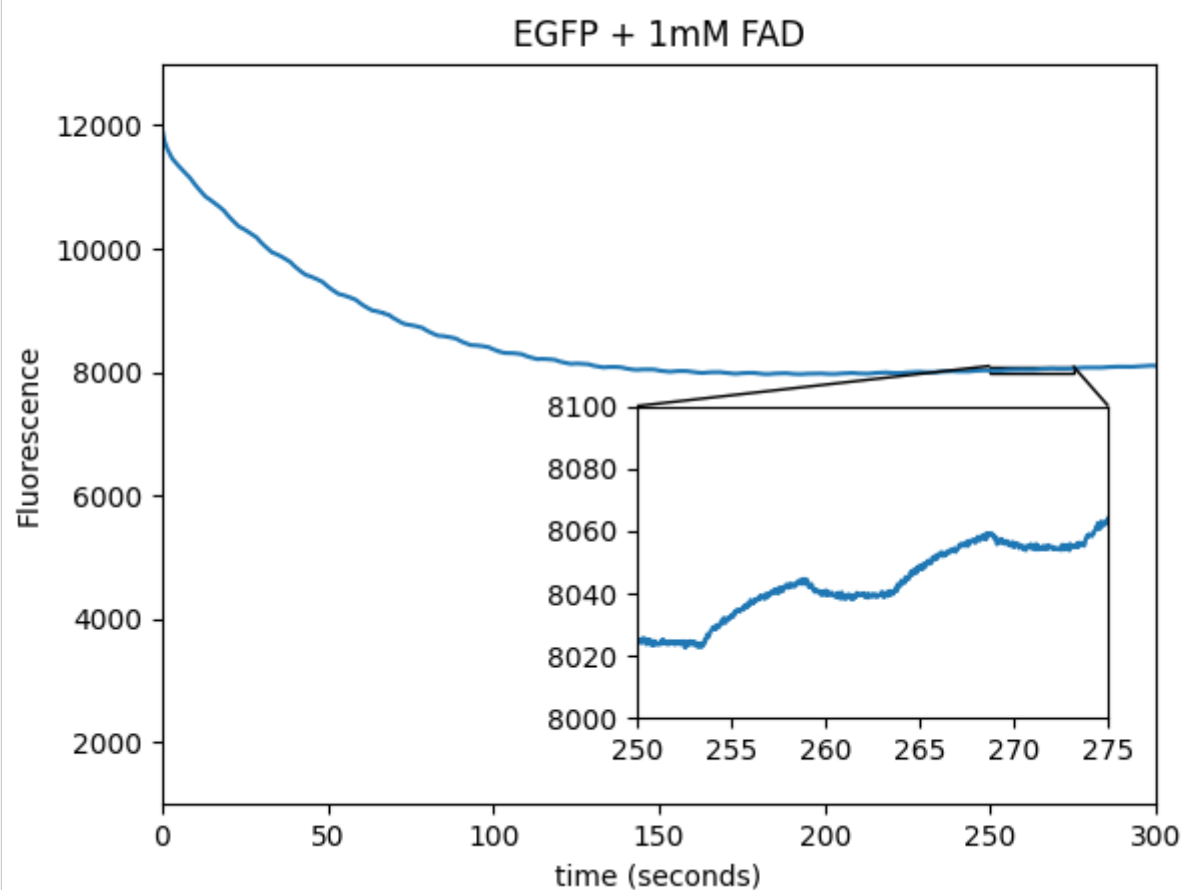
In order to establish that EGFP is indeed the source of the fluorescent magnetoresponse and it is not merely the flavin accumulating on the bead surface, we deleted tyrosine 66 from EGFP; as shown below, this eliminates magnetoresponse in the green channel.

Deletion of the chromophore tyrosine extinguishes the fluorescence while maintaining the overall protein fold, (at least with mVenus, Steven Vogel, personal communication), and we believe it has the same effect on EGFP. We also covalently attached a flavin to the C-terminus of EGFP Δ Y66 and observed no modulation by the magnetic field. Unlike EGFP/FAD mixtures, EGFP/FMN mixtures do not exhibit a magnetic field response, possibly because FMN does not bind EGFP.



Additional controls using purified EGFP in solution

We repeated the experiments from [Figure 1](#) (which used immobilized EGFP on beads) using pure protein in solution (PBS), in wells small enough to be completely covered in an FOV. This prevents diffusion artifacts from complicating the interpretation of the traces. We observed similar results, indicating that the beads are irrelevant to the modulation of fluorescence by the magnetic field.

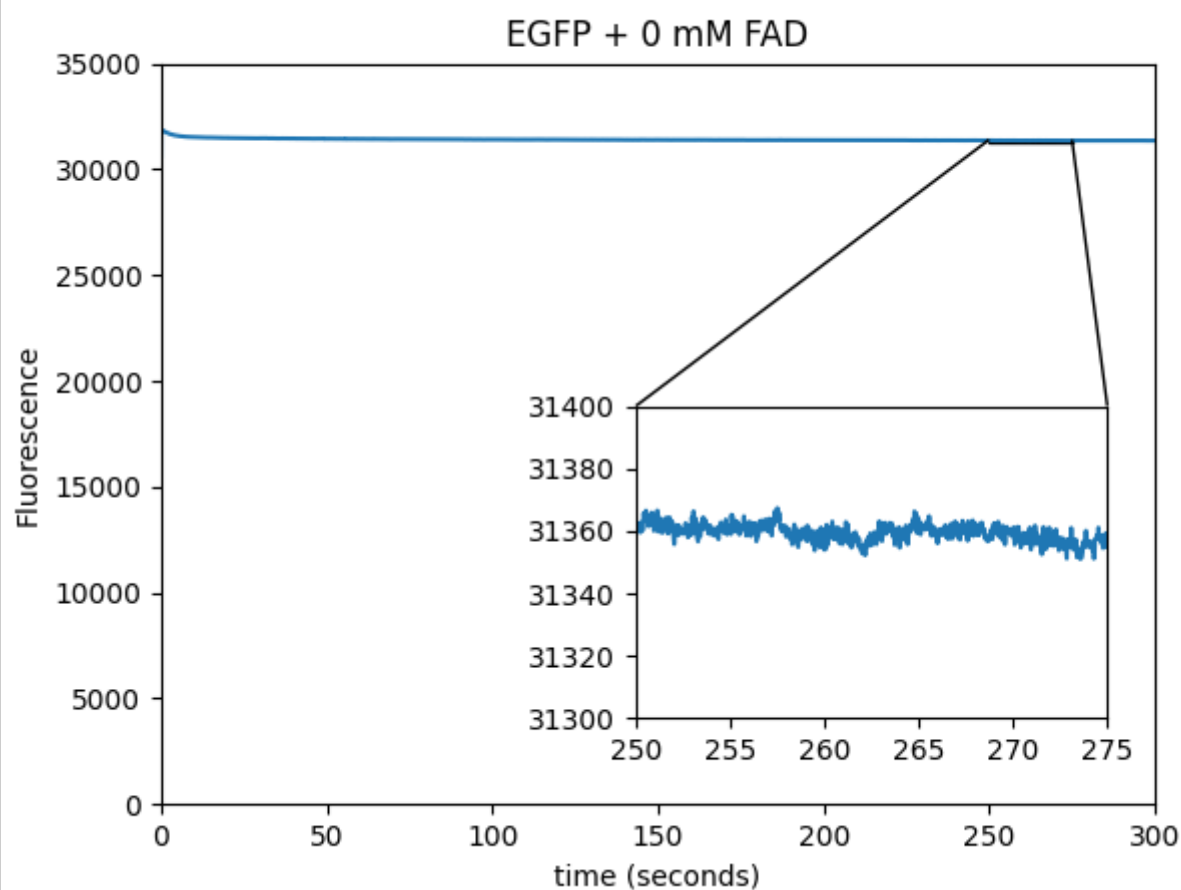


Sample 19.1 μ M EGFP + 1 mM FAD ▼

Like Figure 1 of the main text, but EGFP is in solution instead of attached to Ni-NTA beads or in *E. coli*

Dependence of EGFP magnetofluorescence on FAD concentration

We used purified EGFP in solution to determine the magnetofluorescent response as a function of FAD concentration.



FAD concentration:

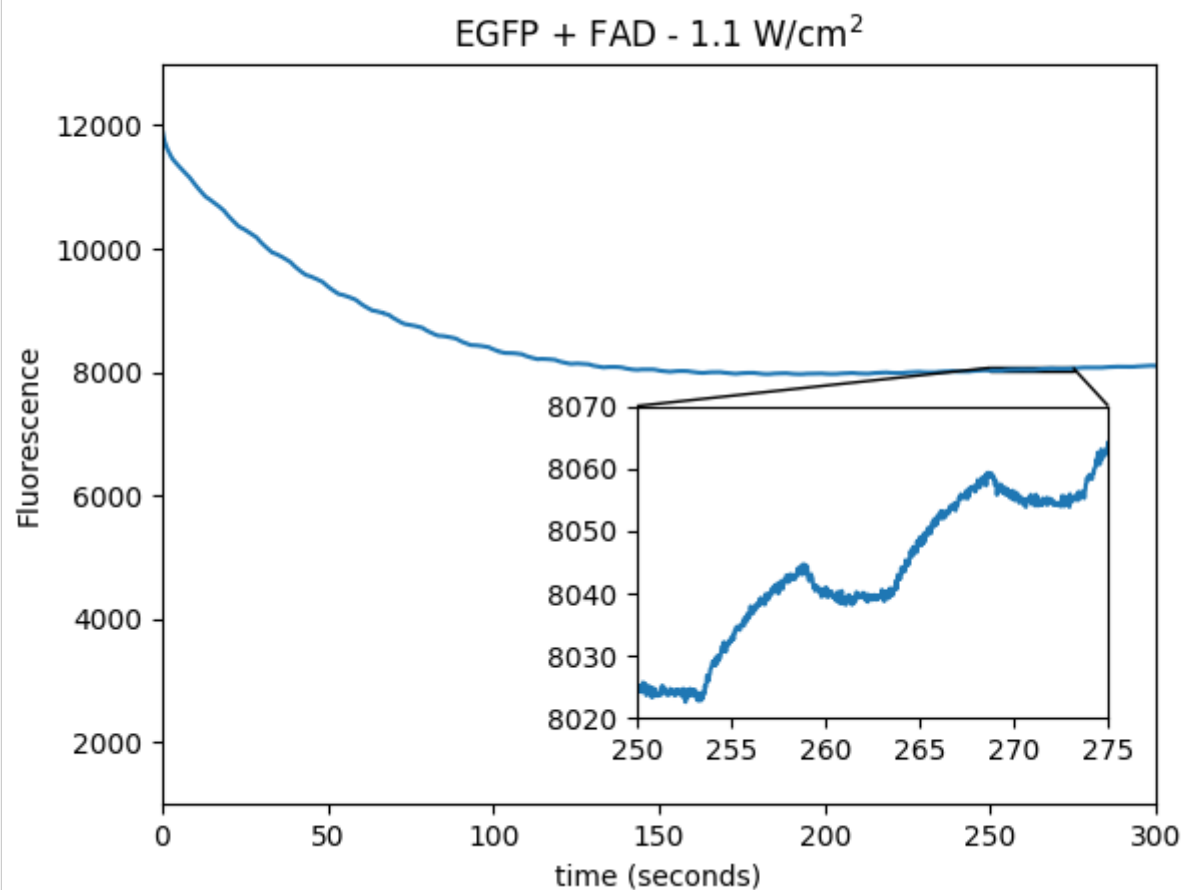
19.1 μ M EGFP alone



The magnetic field response of EGFP fluorescence depends on FAD concentration. We imaged 19 μ M purified EGFP protein in PBS in the presence of increasing concentrations of FAD. The whole well fit in an FOV, allowing us to ignore diffusion. It is apparent that addition of FAD quenches EGFP fluorescence, and is able to elicit a magnetic field response at all concentrations tested, from 250 μ M to 2 mM.

Dependence of EGFP magnetofluorescence on 470 nm power

We used 19.1 μ M EGFP + 1 mM FAD in solution to determine the dependence of the magnetic field modulation of fluorescence as a function of 470 nm excitation power.



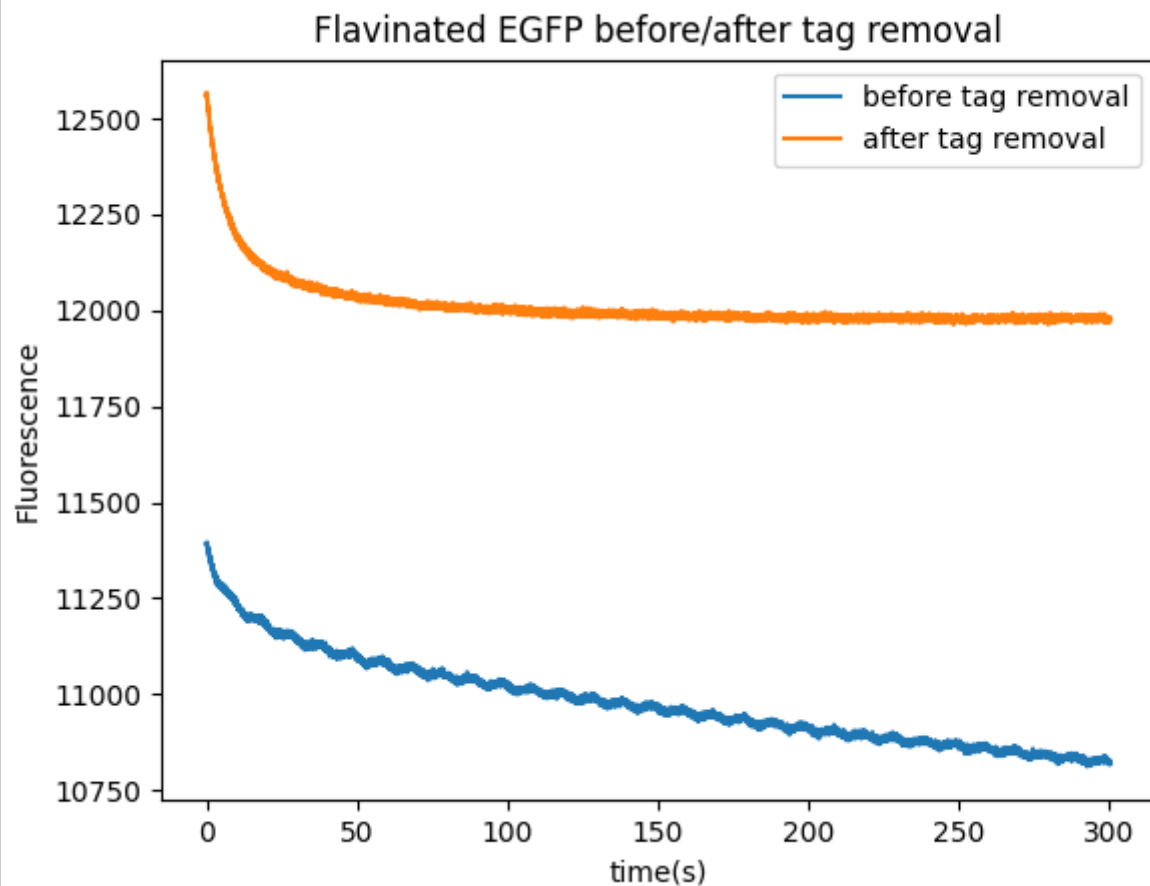
power:

1.1 W/cm² ▼

Magnetic field response of EGFP/FAD fluorescence as a function of excitation power. We imaged 19 μ M EGFP + 1 mM FAD with varying powers. We observed a response at all powers tested.

EGFP-FlavinTag is magnetoresponsive

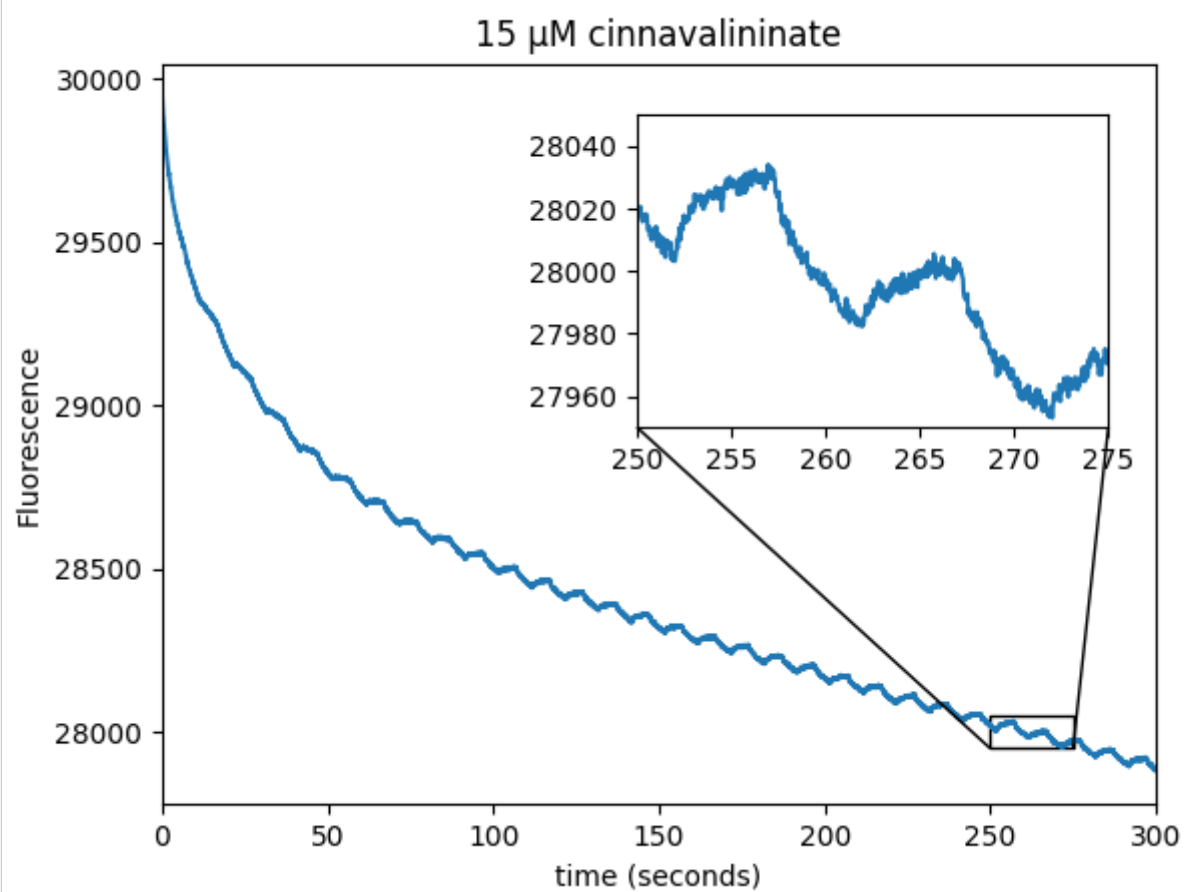
We purified beads with a flavin covalently attached to EGFP by a [FlavinTag](#) sequence and co-expression of the ApbE flavin transferase. The sequence between the EGFP C-terminus and the FlavinTag also encoded for a TEV protease digestion site. This sample was magnetoresponsive, and TEV protease abolishes the response.



EGFP-FlavinTag beads' fluorescence is magnetoresponsive. Beads coated with EGFP with a covalently attached flavin produce fluorescence that is magnetic field sensitive (blue trace). Removal of the FlavinTag using TEV protease renders the beads' fluorescence insensitive to magnetic fields (orange trace). The fluorescence of EGFP increases as we remove the FlavinTag.

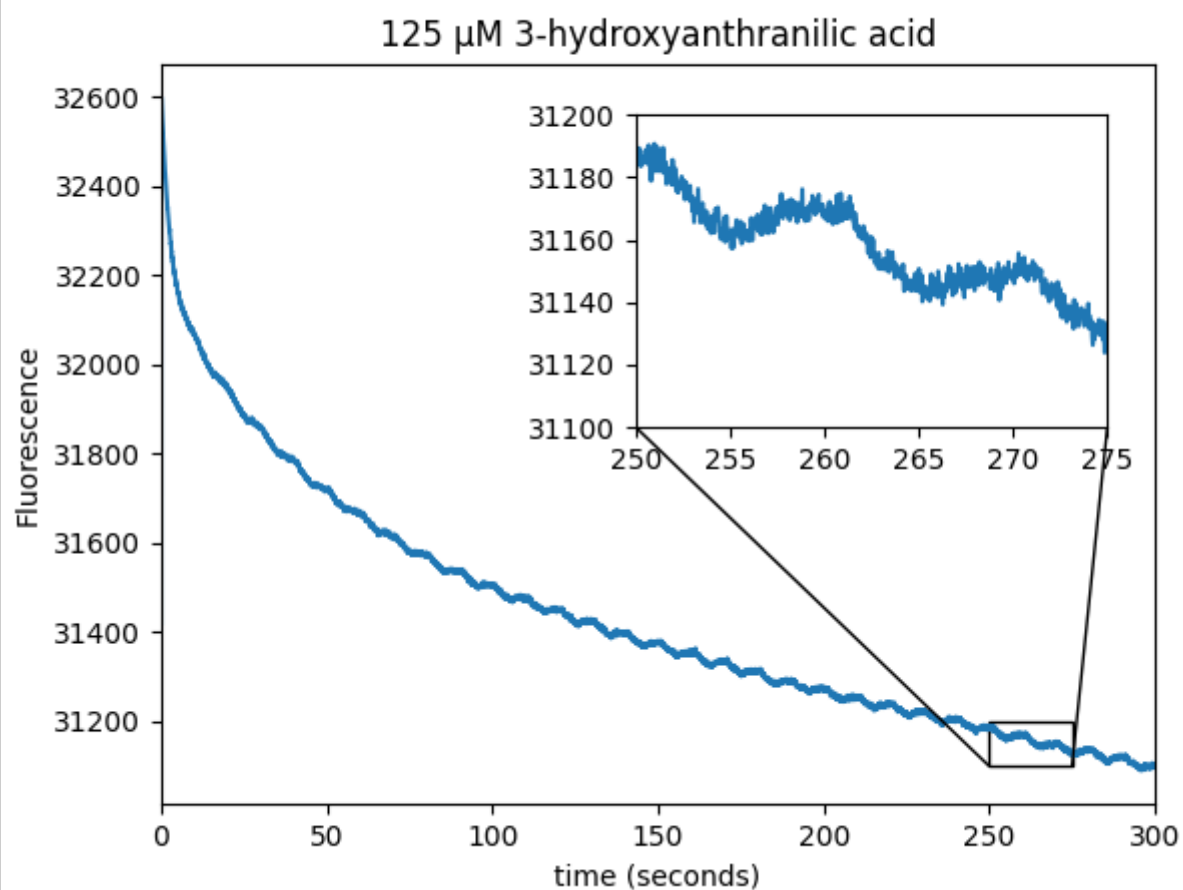
EGFP magnetofluorescence with non-flavin cofactors

We observed that WST-8 dye is able to induce magnetic field modulation of EGFP fluorescence. The metabolite screen also identified several positive hits (cinnavalininate, Hydroxykynurenine, Homogentisic acid, 3-Hydroxyanthranilic acid) as well as recovering FAD as a positive hit.



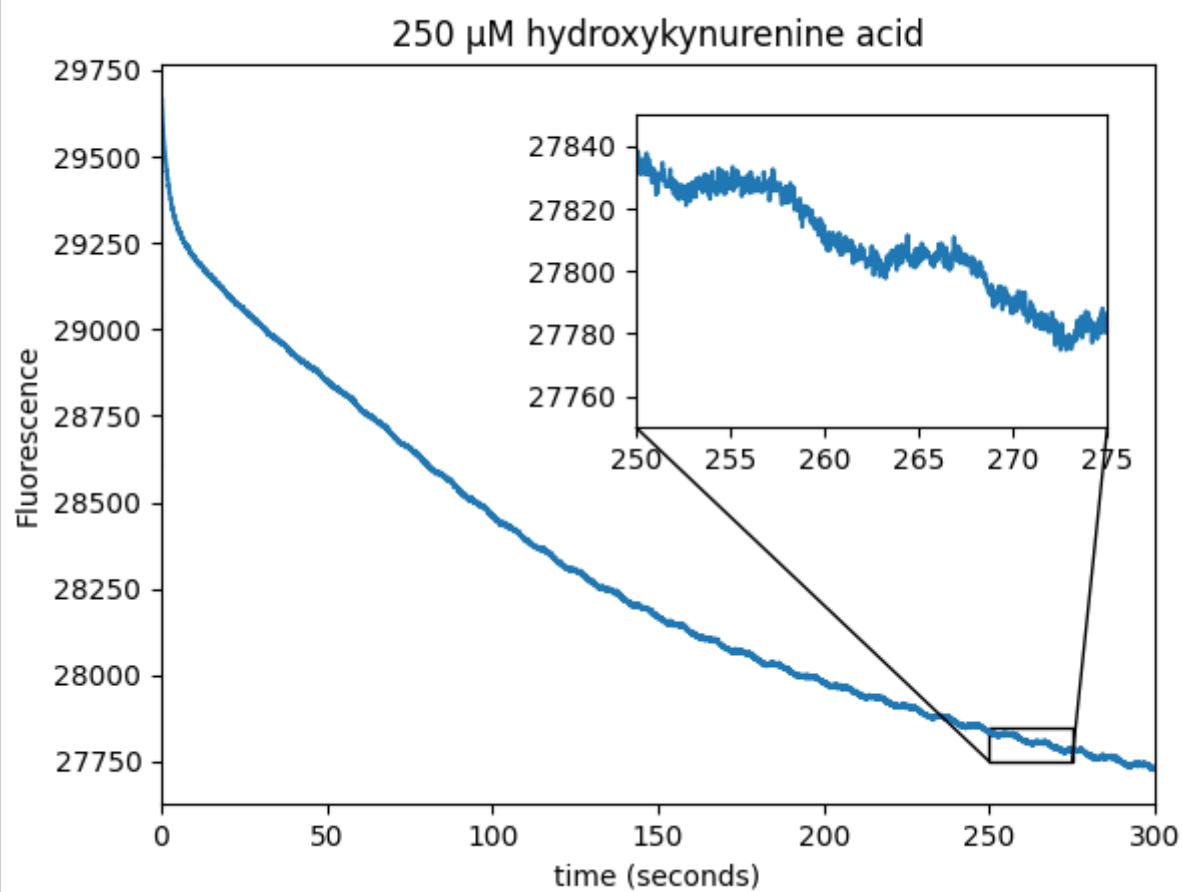
Sample: EGFP + 15 μ M cinnavalininate ▼

Magnetic field response of EGFP/cinnavalininate solutions. We imaged 19.1 μ M EGFP with varying concentrations of cinnavalininate in a 1536-well plate.



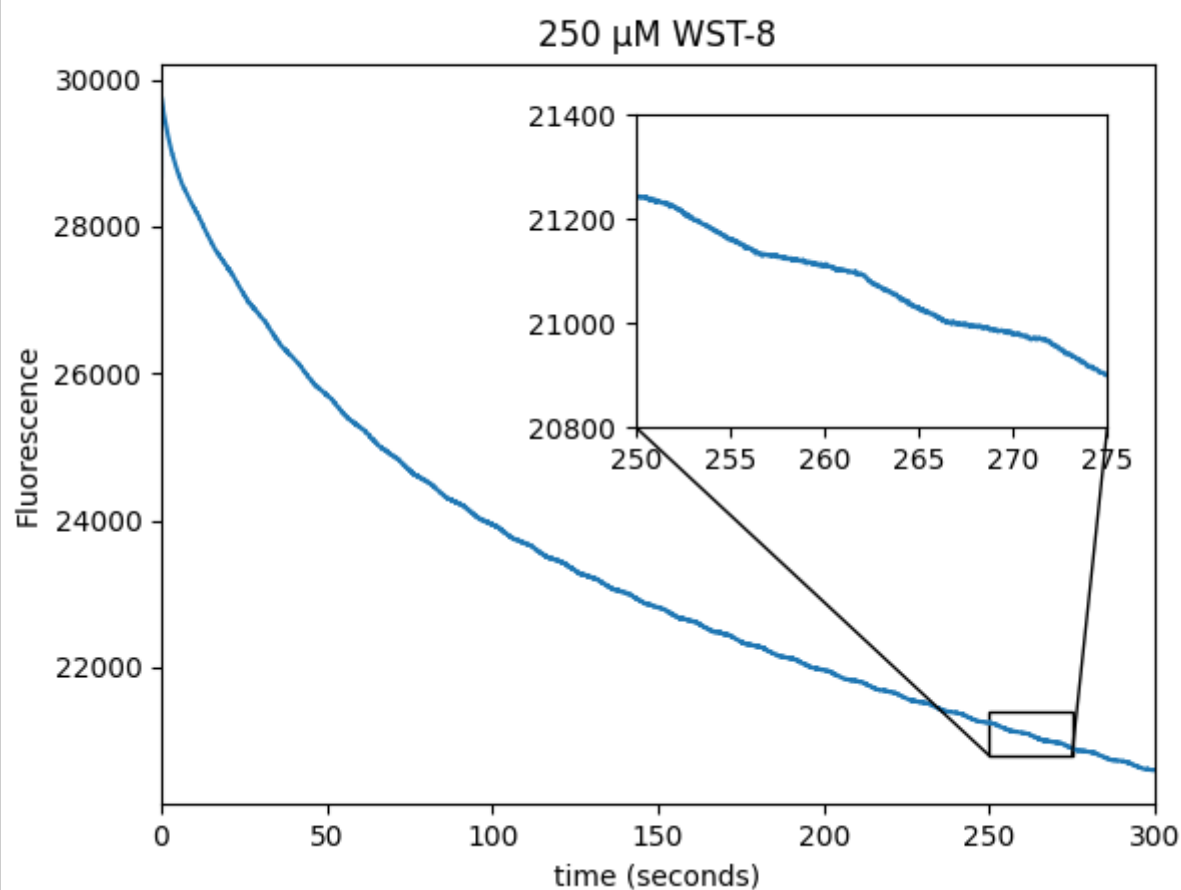
Sample: EGFP + 125 μ M 3-hydroxyanthranilic acid ▼

Magnetic field response of EGFP/3-hydroxyanthranilic acid solutions. We imaged 19.1 μ M EGFP with varying concentrations of 3-hydroxyanthranilic acid in a 1536-well plate.



Sample:

Magnetic field response of EGFP/hydroxykynurenine solutions. We imaged 19.1 μ M EGFP with varying concentrations of hydroxykynurenine in a 1536-well plate.



Sample:

EGFP + 250 μ M WST-8 ▼

Magnetic field response of EGFP/WST-8 solutions. We imaged 19.1 μ M EGFP with varying concentrations of WST-8 in a 1536-well plate.

List of screened compounds:

[screen_compounds.txt](#)

Plasmids and sequences

[pRSETB 6xHis-mEGFP-FlavinTag plasmid Snapgene file](#). *E. coli* plasmid encoding a fusion between 6xHIS-EGFP and the FlavinTag; [plain text format](#)

[pRham-Streptag-APBE plasmid Snapgene file](#). *E. coli* plasmid encoding a flavin transferase; [plain text format](#)

[pCDH EF1 Mito-EGFP-FLVN-T2A-APBE Snapgene file](#). Mammalian cell plasmid encoding EGFP-FlavinTag-T2A-ApbE targeted to mitochondria via fusion to the COX8A presequence; [plain text format](#)

[pRSET-AsLOV\(C450A V416V\) Snapgene file](#). *E. coli* plasmid encoding a mutant of the LOV domain; [plain text format](#)

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6. [Kattnig 2016] Chemical amplification of magnetic field effects relevant to avian magnetoreception; Kattnig, D.R., Evans, E.W., Déjean, V., Dodson, C.A., Wallace, M.I., Mackenzie, S.R., Timmel, C.R. & Hore, P.J.; Nature Chemistry 8 384-391 (2016) <https://doi.org/10.1038/nchem.2447>



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