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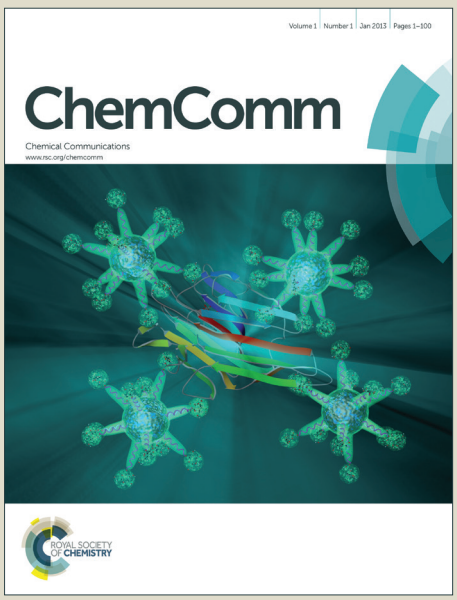





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# Fluorescence-detected magnetic field effects on radical pair reactions from femtolitre volumes

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C. A. Dodson,[‡ae] C. J. Wedge,[‡bf] M. Murakami,[c] K. Maeda,[cdg], M. I. Wallace[a] and P. J. Hore[*c]

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**We show that the effects of applied magnetic fields on radical pair reactions can be sensitively measured from sample volumes as low as ~100 femtolitres using total internal reflection fluorescence microscopy. Development of a fluorescence-based microscope method is likely to be a key step in further miniaturisation that will allow detection of magnetic field effects on single molecules.**

Flavins — tricyclic aza-aromatic compounds — occur widely in Nature and perform a variety of biological functions, many of which involve electron transfer.[1] Attention has recently focussed on the flavoprotein cryptochrome which has been proposed as the primary sensory molecule by which migratory birds detect the direction of the Earth's magnetic field.[2-5] Blue-light excitation of the flavin adenine dinucleotide (FAD) chromophore in cryptochrome is thought to trigger intra-protein electron transfers along a triad of tryptophan (Trp) residues to give a stabilised, charge-separated FAD-Trp radical pair.[6-9] Transient absorption studies[10] have shown that coherent interconversion of the electronic singlet and triplet states of the radical pair gives rise to long lived states of the protein whose quantum yields can be modified by applied magnetic fields via the well-established radical pair mechanism.[11, 12]

Studies of the magnetic sensitivity of cryptochromes are currently constrained by the relatively high protein concentrations and large sample volumes required for absorption-based spectroscopy and by the photo-degradation caused by the intense laser pulses often needed for such measurements.[13, 14] Cryptochromes — in particular the vertebrate proteins — are difficult to express recombinantly in large quantities and tend to aggregate in concentrated solution. Further *in vitro* studies of their magnetic responses will require a detection method compatible with much smaller quantities of protein, the obvious choice being fluorescence.

Fluorescence has been used extensively to monitor magnetic field effects (MFEs) on the photochemistry of small organic radicals. In appropriate, usually non-aqueous, solvents singlet but not triplet radical pairs can often recombine to form an exciplex whose fluorescence provides a convenient and sensitive probe of the magnetic response.[15-18] Effective though such an approach has proved, it is manifestly inapplicable when the radical pair cannot recombine to form a luminescent state. Many radical pairs, including flavin-Trp, do not form excited molecular complexes and cryptochromes have no other electronically excited states that can be populated from the FAD-Trp radical pair.

However, flavins do have fluorescent excited states that can be exploited as MFE probes. For cyclic photochemical reactions under conditions of continuous illumination, the fluorescence intensity reflects the steady state concentration of ground state flavin, which in turn depends on the concentrations of long-lived radical intermediates in the photocycle. If all other species are short lived and thus present in low concentrations, any increase in the steady state concentration of long lived radicals must be balanced by a corresponding decrease in the concentration of the flavin ground state and therefore in the fluorescence. Prompt emission arising from photo-excitation of the ground state is thus an alternative means of studying the effects of applied magnetic fields.

We report here a new method for studying MFEs on flavin photo-reactions using total internal reflection fluorescence (TIRF) microscopy.[19] The advantages include the ability to detect signal from a small area (~30 μm × 30 μm) of a thin layer of solution (~100 nm) and the opportunity to use much smaller quantities of precious samples. Moving to microscopy-based fluorescence

measurements will also be crucial for attempts to measure MFEs on single molecules. To determine whether cryptochromes display the properties expected of biological compass sensors, it will be necessary to characterise their directional responses to weak external magnetic fields. Such an advance is likely to exploit measurements on surface-immobilized single molecules, avoiding the need for bulk alignment of a protein sample.

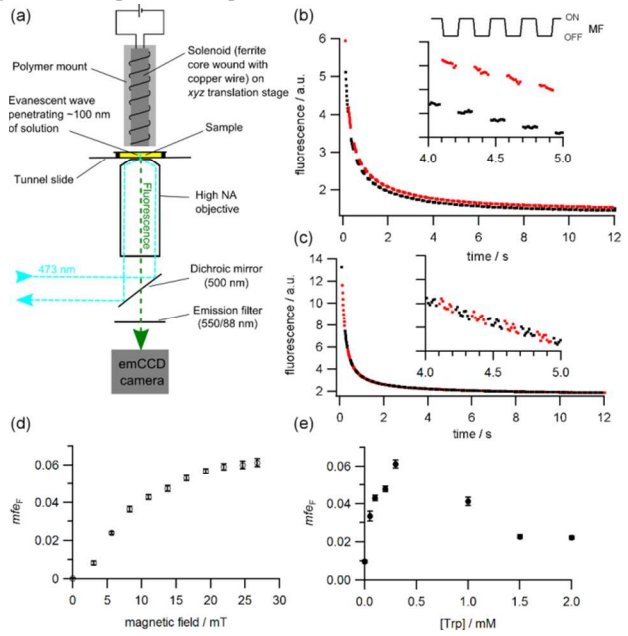


**Fig. 1** Fluorescence detection of MFEs. (a) Schematic diagram of the experiment. (b) Fluorescence signals from 1 μM FAD + 300 μM Trp solutions. Red/black: data measured in absence/presence of a 26.8 mT magnetic field. A portion of the signal is shown inset. (c) As b but 1 μM FMN without Trp. (d) Fractional MFE (1 μM FAD + 300 μM Trp) as a function of $B_0$. (e) Fractional MFE (1 μM FAD) as a function of Trp concentration for $B_0$ = 26.8 mT.

In this preliminary communication, we report TIRF measurements of MFEs on both flavin-adenine and flavin-Trp radical pairs formed, respectively, by intra- and intermolecular electron transfer reactions of photo-excited triplet FAD.[20, 21]

The fluorescence of FAD-Trp solutions at low pH, generated by a 473 nm continuous-wave laser, was monitored by TIRF microscopy (Fig. 1a) in the presence of a square-wave modulated static magnetic field. Fluorescence was detected from a ~100 fL volume of solution (see Materials and Methods, ESI† for details). The switched magnetic field produced a clear stepwise modulation of the fluorescence (Fig. 1b) with a significant reduction in the emission at the beginning, and a comparable increase at the end, of each field pulse. This is as expected for a triplet-born radical pair: the Zeeman interaction of the electron spins in the radical pair isolates the $T_{+1}$ and $T_{-1}$ triplet states from the $T_0$ triplet and S (singlet) states and so inhibits the formation of singlet radical pairs and therefore, also, the return to the ground state.[20] Similar fluorescence-detected MFEs were observed for FAD in the absence of Trp, but not for controls containing flavin mononucleotide (FMN), which does not contain the adenine electron donor (Fig. 1c), or fluorescein or Alexa Fluor® 488 which would not be expected to show a MFE (Fig. S1, ESI†).

Magnetic field effects were quantified by means of the parameter $mfe_F = [F(0) - F(B_0)]/F(0)$ (see ESI† for details). $F(B_0)$ and $F(0)$ are the fluorescence intensities during the periods when the magnetic field is, respectively, present (at strength $B_0$) and absent (black and red sections of Fig. 1b) . This fractional MFE increased with $B_0$, tending to level out, as expected,[20] when the applied field exceeded the effective hyperfine interaction of the radical pair (Fig. 1d). As the Trp concentration was increased in the range 0–300 μM, $mfe_F$ rose from 1% to 6% and fell back to 2% at ~2 mM (Fig. 1e).

The field-induced changes in FAD fluorescence were observed on a background of steadily decreasing overall fluorescence (Fig. 1b). This decay was also observed for FMN (Fig. 1c) and fluorescein (Fig. S1, ESI†) and is attributed, at least in part, to photobleaching.

To validate our TIRF approach and to gain more mechanistic information, we studied the same FAD-Trp solutions by transient absorption in order to distinguish the various states of the reactants and their kinetics. The neutral, protonated flavin radical, $FADH^{\bullet}$, is characterised by a broad absorption in the range 500–600 nm overlapping that of the Trp cation radical ($Trp^{\bullet+}$); the absorption of the excited triplet flavin, $^3FAD$, extends to longer wavelengths, 500–700 nm (Fig. S2, ESI†). FAD itself absorbs at wavelengths below 500 nm and Trp is transparent at wavelengths above ~300 nm.[22]

The transient absorption signals of FAD-Trp solutions monitored at 580 nm (where $Trp^{\bullet+}$, $FADH^{\bullet}$ and $^3FAD$ have comparable extinctions) showed a biphasic decay with the lifetime of the slow component increasing significantly in the presence of Trp (Fig. 2a). The time-dependent absorption at 650 nm, where $^3FAD$ is the dominant contributor, was also biphasic (Fig. 2b). In the absence of Trp, the signals at the two wavelengths (red traces in Figs 2a & b) are very similar and differ only in amplitude, reflecting the wavelength-dependence of the extinction coefficients. This indicates that the decay of $^3FAD$ in the absence of Trp also dominates the 580 nm signal in agreement with Murakami et al.[21] In the presence of Trp, the absorbance at 650 nm was rapidly quenched (Fig. 2b) and the amplitude of the slow phase observed at 580 nm increased (Fig. 2a). We therefore assign the slow decay at 580 nm to long lived $FADH^{\bullet}$ and/or $Trp^{\bullet+}$ radicals formed by $^3FAD$ + Trp reaction.

MFE time-profiles were calculated as the change in the transient absorbance produced by an applied magnetic field: $\Delta\Delta A = \Delta A(B_0) - \Delta A(0)$. $\Delta\Delta A_{540\text{-}600}$ data for $B_0$ = 200 mT, averaged over the wavelength range 540–600 nm, showed a fast component at Trp concentrations below ~0.1 mM and a much slower component at higher concentrations (Fig. 2c and Fig. S3, ESI†). At longer wavelengths (630–710 nm) the fast component dominated and its amplitude and lifetime decreased markedly with increasing Trp concentration (Fig .2d). The MFE detected by absorption is positive, i.e. $\Delta A(B_0) > \Delta A(0)$, consistent with the negative effect observed by fluorescence, $F(B_0) < F(0)$.

$\Delta\Delta A_{630\text{-}710}$ is dominated by magnetically sensitive changes in the population of $^3FAD$ while $\Delta\Delta A_{540\text{-}600}$ monitors $FADH^{\bullet}$ and $Trp^{\bullet+}$ as well as $^3FAD$. In the absence of Trp (red traces, Fig. 2c and d), $^3FAD$ is, once again, the major contributor to the signals at both wavelengths. The changes brought about by the addition of Trp are attributed to the reaction of $^3FAD$ with Trp to form long lived $FADH^{\bullet}$ and $Trp^{\bullet+}$ radicals (slow components in Fig. 2c and Fig. S3, ESI†).

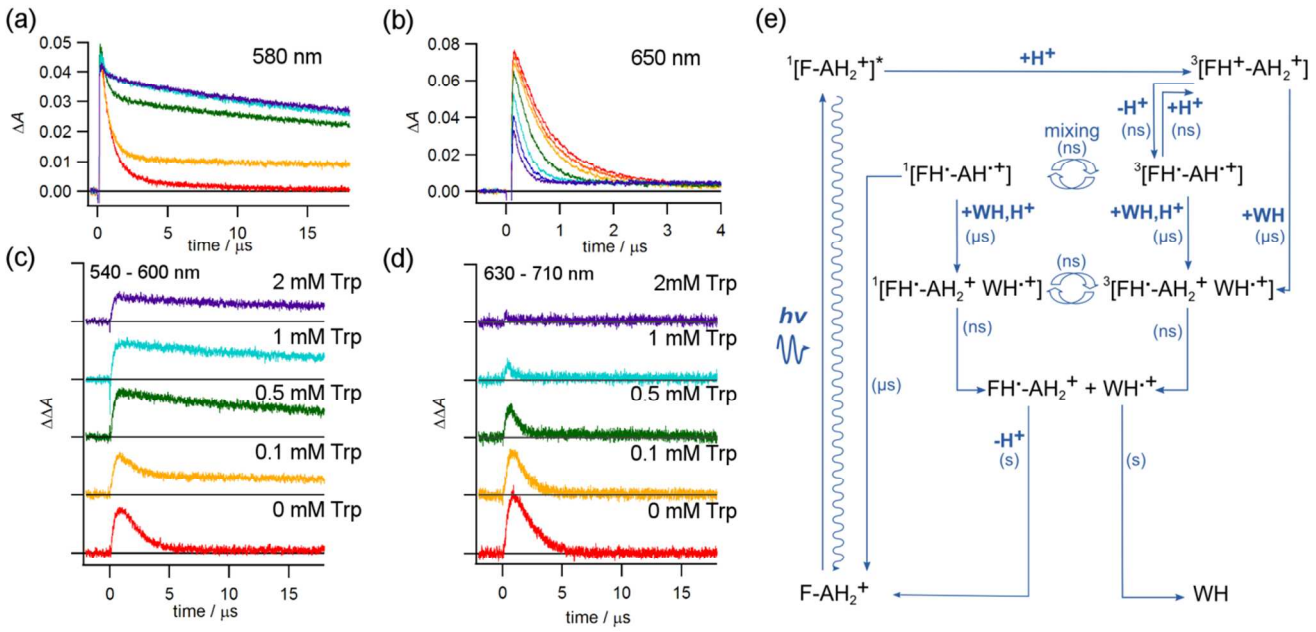


**Fig. 2** Transient absorption detection of MFEs. Time-dependence of the absorbance change at (a) 580 nm and (b) 650 nm. The colour code for the Trp concentrations in (a) and (b) is the same as in (c) and (d). MFE time-profiles averaged over the wavelength ranges (c) 540–600 nm and (d) 630–710 nm. (e) FAD photocycle including the FAD + Trp reaction. FAD is shown as F–$AH_2$ with the flavin (isoalloxazine) and adenine groups denoted F and A. Trp is denoted W.

The transient absorption measurements summarized in Fig. 2a-d enable us to extend the previously published FAD photocycle[21] to include the FAD + Trp reaction (Fig. 2e). Blue-light illumination of ground state FAD produces the excited singlet state which either fluoresces or intersystem crosses to $^3$FAD. Electron transfer from the adenine to the isoalloxazine of FAD in $^3$FAD forms a geminate triplet flavin-adenine radical pair which undergoes magnetically sensitive triplet ↔ singlet interconversion. The rapid reversibility of the electron transfer step is indicated by the MFE on $^3$FAD as observed previously.[21]

Reverse electron transfer in the singlet state of the flavin-adenine radical pair provides a return route to ground state FAD. Additionally, $^3$FAD can be quenched by Trp to form an intermolecular radical pair whose constituents ($FADH^{\bullet}$ and $Trp^{\bullet+}$) either undergo reverse electron transfer or diffuse apart to form the long lived free radicals that are responsible for the slow transient absorption components in Figs 2a and c. Due to the fast interconversion between $^3$FAD and the flavin-adenine radical pair, we cannot exclude the possibility that Trp reacts with the pre-existing intramolecular radical pair as well as with the triplet flavin.

As argued above, for a photocycle of the type shown in Fig. 2e, any field-induced increase in the steady state concentration of the long lived radicals is matched by a corresponding decrease in the concentration of the ground state molecules. This point can be seen clearly by comparing Figs 3a and c which show, respectively, $\Delta A(B_0) - \Delta A(0)$ and $F(0) - F(B_0)$. Both absorption- and fluorescence-detected MFEs show the same trend with increasing Trp concentration. Similarly, the increase in the yield of long lived radicals observed by transient absorption (Fig. 3b) is complemented by a decrease in the ground state concentration observed by fluorescence (Fig. 3d).

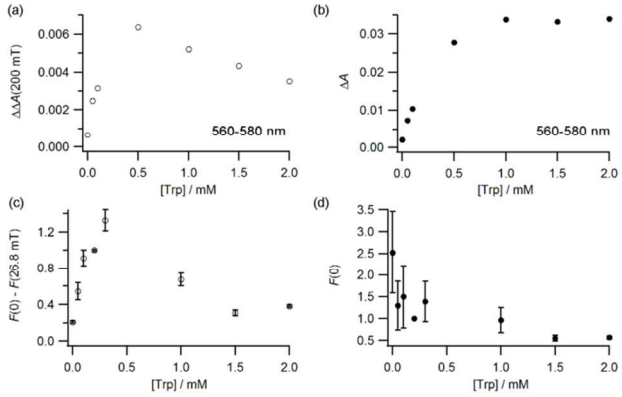


**Fig. 3** Comparison of absorption- and fluorescence-detected MFEs. (a) Transient absorption difference signal, $\Delta\Delta A = \Delta A(200\text{ mT}) - \Delta A(0)$. (b) Transient absorption signal in the absence of a magnetic field, $\Delta A(0)$. (c) Fluorescence difference signal $F(0) - F(26.8\text{ mT})$. (d) Initial fluorescence intensity in the absence of a magnetic field. The data in panels (a) and (b) are averages over the wavelength range 560–580 nm and the time period 5–10 μs. The data in panels (c) and (d) have been normalised to the measurement at 200 μM Trp and error bars show SEM. The FAD concentrations were 0.2 mM (absorbance) and 1 μM (fluorescence).

We have demonstrated for the first time that MFEs can be measured by TIRF microscopy and that flavin fluorescence probes the same magnetically-sensitive reaction steps as optical absorption of the transient radical intermediates. Fluorescence measurements are highly sensitive and thus suitable for much lower concentrations than absorption-based techniques, making them attractive for samples that are prone to concentration-dependent aggregation. By using an inverted microscope with a high numerical aperture objective and TIR illumination we have reduced the volume probed to ~100 fL. Together, these developments reduce the total sample requirement by several orders of magnitude, opening the possibility of measuring MFEs on samples for which low yields or concentrations previously

made measurements impractical. Despite the low quantum yield of FAD fluorescence in cryptochromes (due to rapid electron transfer from the Trp triad), we believe it will be possible to study MFEs on the intra-protein FAD-Trp radical pairs. The miniaturisation afforded by fluorescence microscopy is also an important step on the journey towards the goal of magnetic field effect measurements on single molecules.

Like previous TIRF measurements on FAD[23], our method already possesses single-molecule sensitivity. However, further improvements will be needed for single-molecule MFE detection: either to resolve time-dependent switching between states, or to increase the precision in measurement of the mean photon emission rate during the photocycle. These advances could be achieved either by improvements in the time-resolution of the experiment, or modification of the photocycle kinetics.

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## Notes and references

[a] Department of Chemistry, University of Oxford, Chemistry Research Laboratory, 12 Mansfield Road, Oxford OX1 3TA, UK.
[b] Department of Chemistry, University of Oxford, Physical and Theoretical Chemistry Laboratory, South Parks Road, Oxford OX1 3QZ, UK. E-mail: peter.hore@chem.ox.ac.uk
[c] Department of Chemistry, University of Tsukuba, 1-1-1 Tenno-dai, Tsukuba City, Ibaraki Pref, 305-8571, Japan.
[d] Centre for Advanced Electron Spin Resonance, Department of Chemistry, University of Oxford, Oxford OX1 3QR, UK.
[e] Current address: Molecular Medicine, National Heart & Lung Institute, Imperial College London, SAF Building, London SW7 2AZ, UK.
[f] Current address: Department of Physics, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, UK.
[g] Current address: Department of Chemistry, Graduate School of Science and Engineering, Saitama University, 255 Shimo-Okubo, Sakura-ku, Saitama City, Saitama 338-8570, Japan.

‡ These authors contributed equally to this work.
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