Genetically targetable and colour-switching fluorescent probes

Dmytro A Yushchenko[*], *Ming Zhang*, *Qi Yan*, *Alan S Waggoner*, *and Marcel P Bruchez*[*]

Molecular Biosensor and Imaging Center, Department of Chemistry, Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213, USA

E-mail: ydmytry@gmail.com; bruchez@cmu.edu

Contents

General remarks

Standard chemicals were purchased from Sigma. 5/6-carboxy-tetramethyl-rhodamine succinimidyl ester (TMR-NHS) was ordered from Pierce Biotech Inc (Product# 0046406).

HPLC The purity of all dyes described in this article was confirmed by analytical HPLC (Waters Delta 600 System), reversed-phase column: YMC Pack ODS-AQ (10 x 250 mm, # AQ12S05-2510WT) flow rate: 3 ml/min, gradient (A = H_2O, 0.1%TFA and B = CH3CN): A/B 90:10 for 5 min, to A/B 10:90 in 30 min, detection: 550 and 610 nm). Most of the dyes were purified by preparative HPLC (Waters Prep LC 4000 System), reversed-phase column: Waters C18 (19 x 300 mm), flow rate: 10 ml/min.

Absorbance spectra were taken on a Perkin Elmer Lambda45 spectrophotometer. Concentrations of the dyes were measured in Ethanol+5% AcOH. Extinction coefficient of MG dye 80000 and for TMR 105000 was used.

Buffer system. For spectroscopic and microscopy studies a modified phosphate buffered saline system (PBS^+) was used in all experiments unless noted (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, 2mM EDTA, 0.1% w/v Pluronic F-127 (Anatrace), pH 7.4) [1]

Fluorescence spectra were taken on a Quantamaster monochromator fluorimeter (Photon Technology International). Prior to taking spectra usually ~500 nM of the dye was equilibrated with (or without) 1.5 µM dL5 138 FAP [Chris Szent-Gyorgyi, manuscript in prep] for at least 2 hrs at RT. Spectra were corrected for PBS^+ background and wavelength dependent photomultiplier sensitivity.

Quantum yields (QYs) were determined using Cresyl Violet (Kodak, #41830-80-2) as a reference dye with excitation at 515 nm (Table 1) with QY in MeOH 55% [2]

Fluorogen Activating Protein (FAP). For cuvette measurements a dimeric dL5-138 (E52D L91S) mutant was used. The detailed description of this protein will be presented in [Chris Szent-Gyorgyi, manuscript in prep]. In cellular experiments either dL5 FAP was expressed as a fusion protein to the transmembrane domain of B7-1 protein at the inner part of the cellular membrane in HEK 293 cells or dH6 FAP was expressed being fused to the filament forming protein actin (see below).

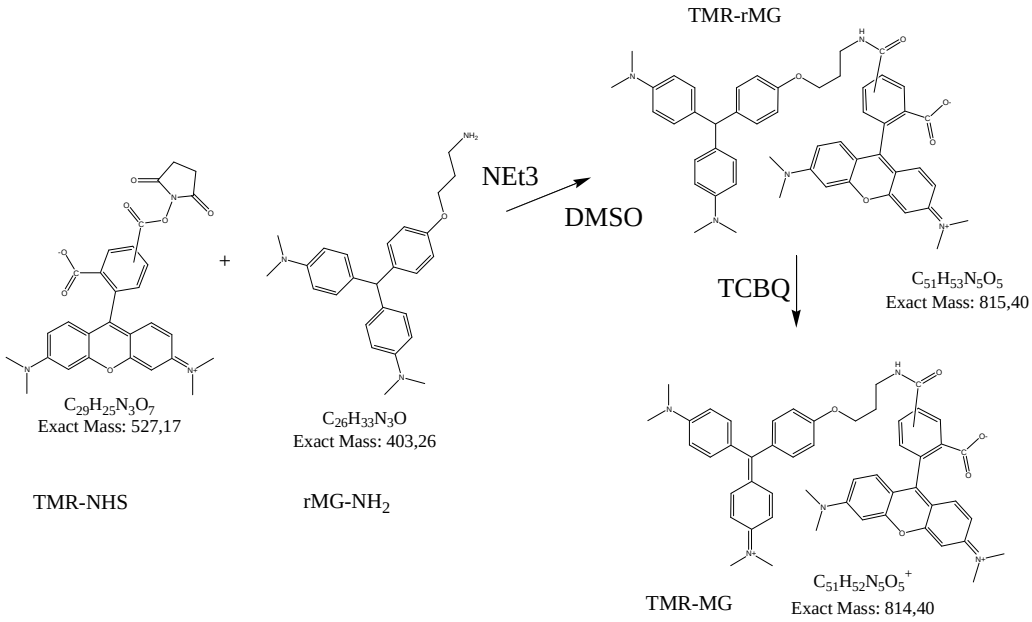
NMR spectra were recorded with Bruker Avance 500MHz spectrometer. All spectra are referenced to the signals of the residual protons of CHD2OD (3.31 ppm) in CD3OD, HOD (4.79 ppm) in D2O or [D_5]DMSO (2.50 ppm) in [D_6]DMSO.

Mass spectra (electro spray ionization, ESI) were obtained with ESI-Ion Trap-MS spectrometer.

Microscopy: All images were taken on a Carl Zeiss LSM-510_META_MP_ConfoCor 3 Confocal Microscope: (See Table 2 for typical settings) or Andor Revolution XD System with Spinning Disk Confocal. Image analysis using the native 12-bit dynamic range of the microscope was carried out with Zeiss ZEN 2009 software or/and ImageJ software. Prior to imaging cells were incubated with the corresponding dye for 20-30 min, time that is sufficient for TMR-MG dyes to penetrate through the plasma membrane and to stain target sites (actin filaments or membrane tethered FAP) and mitochondria. In most cases the concentration of TMR-MG dyes was 150 nM.

Synthesis of TMR-MG dyes

The TMR-meta-MG and TMR-para-MG dyes were synthesized according to scheme 1.



Scheme 1. Scheme of synthesis of TMR-*meta*-MG and TMR-*para*-MG dyes:

Preparation of MG-NH_2 is described in SI of [3] . TMR-NHS (a mixture of two isomers) is a commercially available dye and was ordered from Pierce Biotech Inc (Product# 0046406).

Reaction of TMR-NHS with MG-NH_2 gives TMR-MG with a very low yield. TMR-NHS reacts however much better with the reduced form of MG-NH_2 (rMG-NH_2, a precursor of MG-NH_2). We used rMG-NH_2 to synthesize TMR-rMG (a mixture of isomers) which was then oxidized in the presence of tetrachlorobenzoquinone (TCBQ) to give a mixture of TMR-*meta*-MG and TMR-*para*-MG (Scheme 1). Two isomers were then separated by HPLC. They were further distinguished and attributed to *meta* and *para* isomers by the chemical shifts of their three aromatic protons in the region 7.5-9 ppm according to [4] and by the NOESY coupling of aromatic protons in TMR moiety (see below).

Protocol:

In a dry (5 ml) round flask, flushed with Ar and containing a stirrer bar, rMG-NH_2 (7.5 mg, 18.6 µmol) was dissolved in dry DMSO (0.5 ml). Then NEt_3 (20 µl, 150 µmol) and TMR-NHS (7.5 mg, 14.2 µmol, mixture of isomers) in DMSO (0.5 ml) was added. The reaction mixture was left stirring overnight at room temperature. Next day an aliquot (1 µl) for HPLC and an aliquot (1 µl) for mass spec were taken in order to check formation of TMR-rMG (Figure 1). After positive mass spec TCBQ (25 mg, 100 µmol) was added to the reaction mixture (which changes colour from pink to violet) and then the reaction mixture was left stirring overnight at room temperature. Next day TMR-MG isomers were purified and separated by prep HPLC.

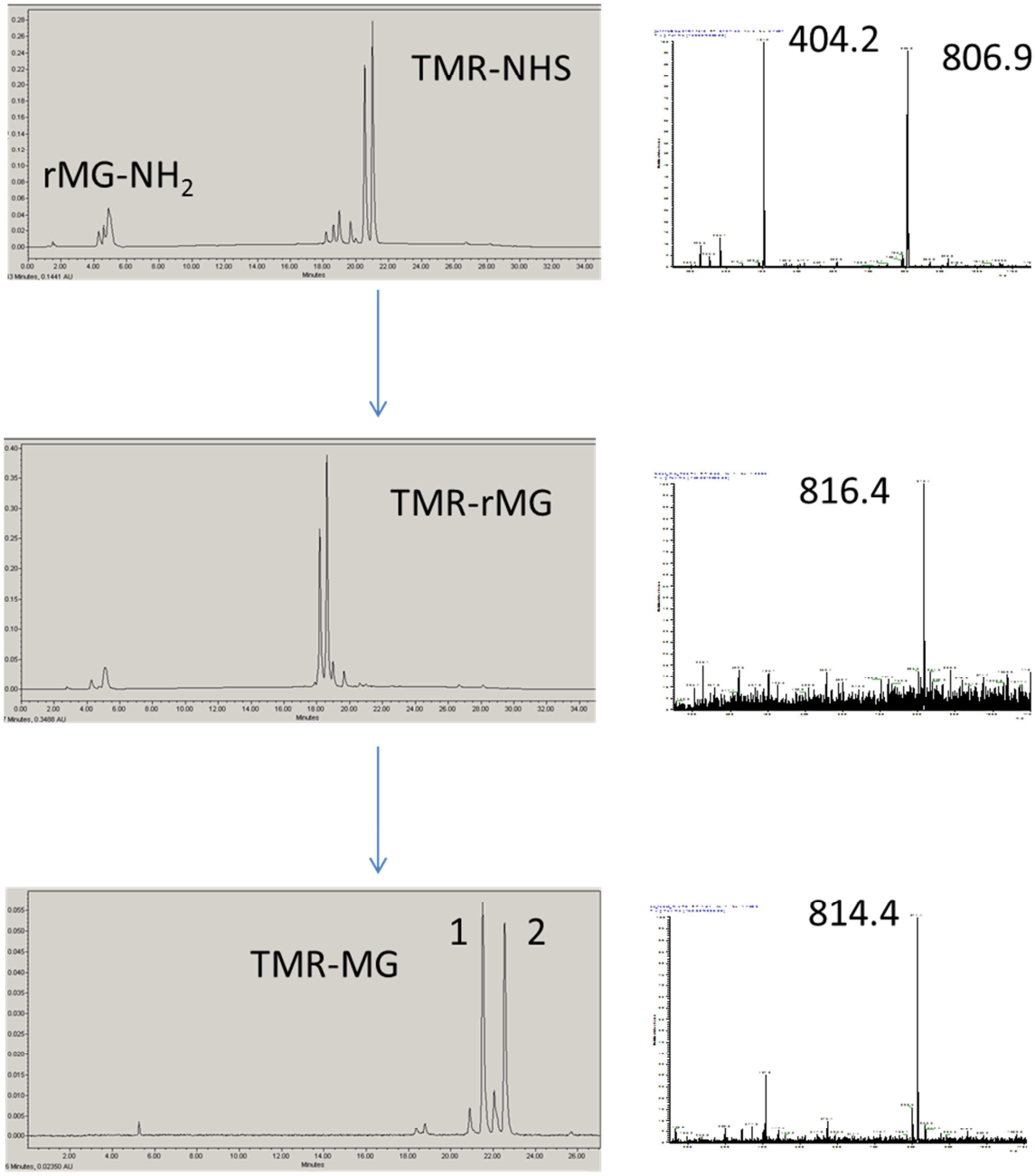
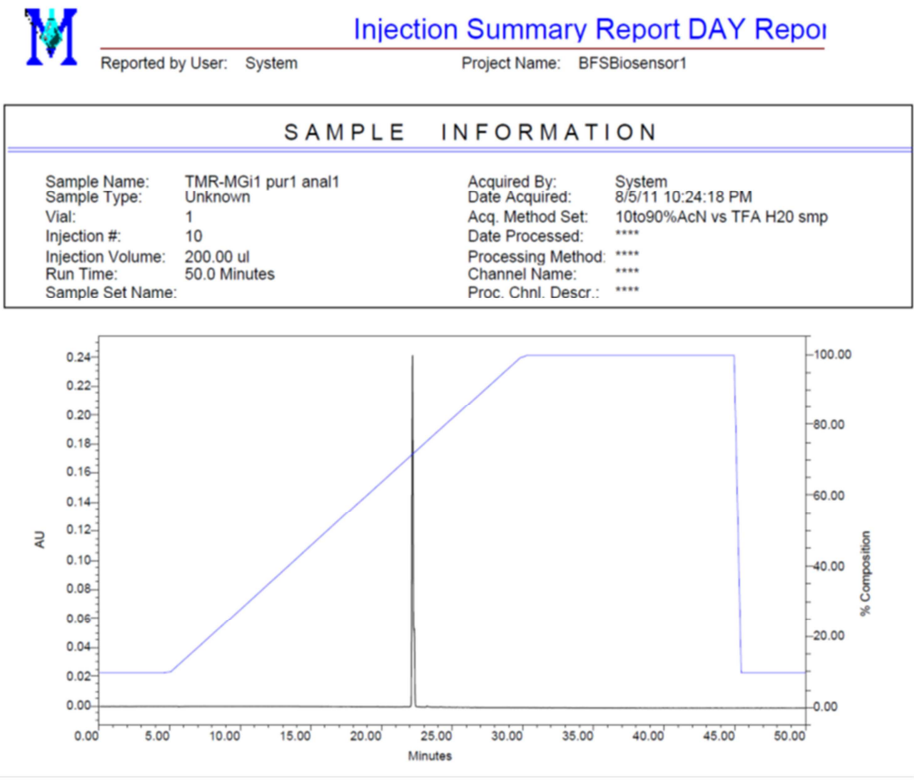


Figure 1. Synthesis of TMR-MG dyes monitored by HPLC and mass spec.

TMR-*meta*-MG

HPLC: Retention time 22.1 min.



Mass spec of TMR-*meta*-MG:

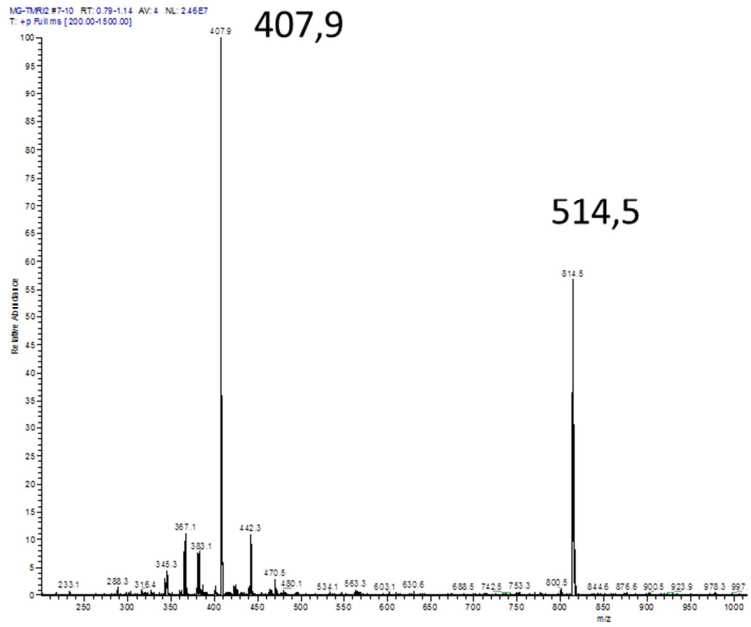


Figure 2. HPLC chromatogram and mass spectrum of TMR-*meta*-MG dye (double purified)

TMR-*meta*-MG (Isomer 1): ^{1}H NMR (MeOD, 500 MHz, ppm), δ = 2.19 (t, *J* = 5.6, 2H), 3.29 (s, 9H), 3.69 (t, *J* = 6.3, 2H), 4.30 (t, *J* =4.8, 2H), 6.89 (s, 2H), 7.03-7.18 (m, 12H), 7.38 (d, *J* =7.6, 4H), 7.60 (s, 1H), 8.19 (d, *J* = 7.2, 1H), 8.40 (d, *J* =8.5, 1H), 8.87 (bs, 1H); m/z = 514,5

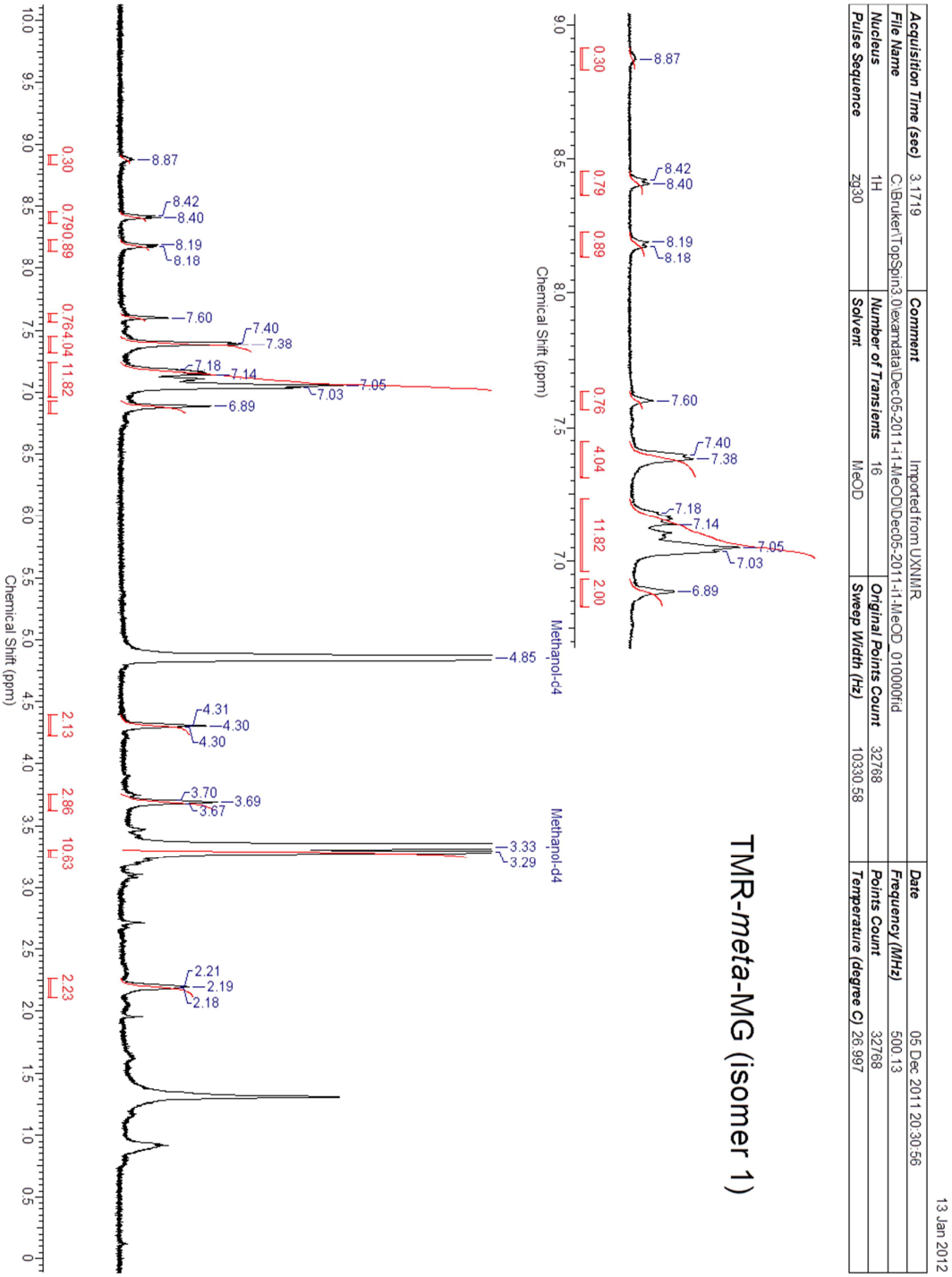
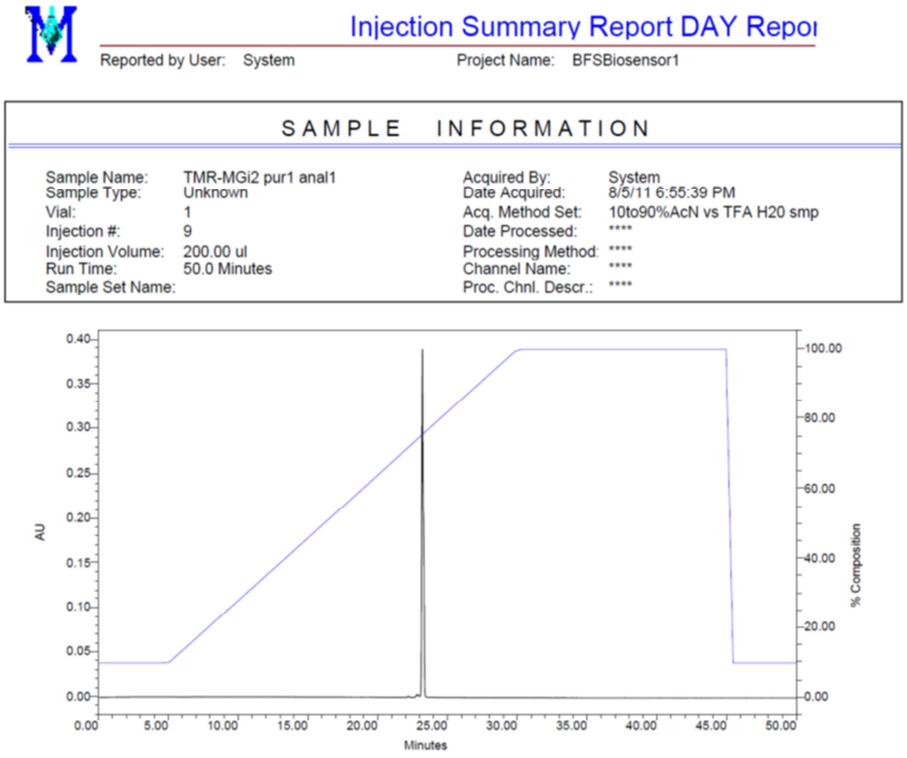


Figure 3. ^{1}H NMR (500 MHz in MeOD) spectrum of TMR-*meta*-MG dye (single purified)

TMR-*para*-MG

HPLC: Retention time 23.2 min.



Mass spec of TMR-*para*-MG:

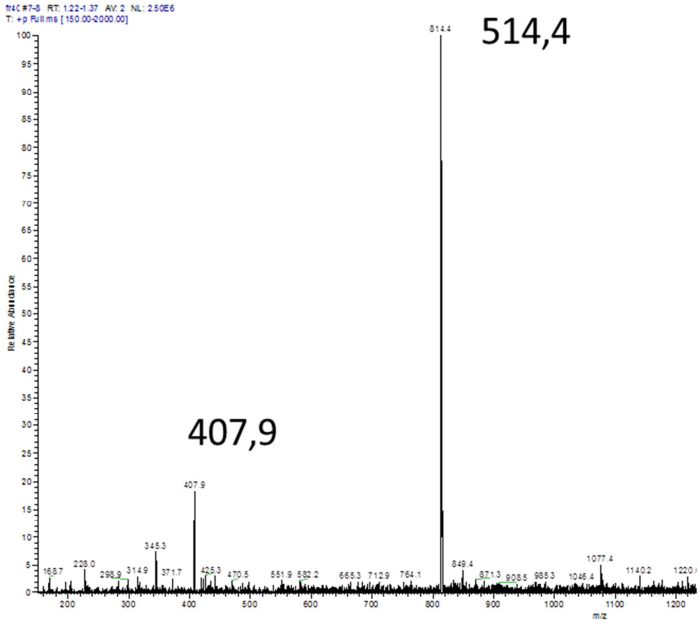


Figure 4. HPLC chromatogram and mass spectrum of TMR-*para*-MG dye (double purified)

TMR-*para*-MG (Isomer 2): ^{1}H NMR (MeOD, 500 MHz, ppm), δ = 2.28 (t, *J* = 5.7, 2H), 3.75 (t, *J* = 6.0, 2H), 4.36 (t, *J* =5.6, 2H), 7.01-7.06 (m, 8H), 7.15 (d, *J* = 9.1, 2H), 7.22 (d, *J* =8.2, 2H), 7.39 (d, *J* =7.9, 2H), 7.44 (d, *J* =8.5, 4H), 7.57 (d, *J* =7.5, 1H), 8.30 (d, *J* = 6.6, 1H), 8.80 (s, 1H), 9.03 (bs, 1H); m/z = 514,4

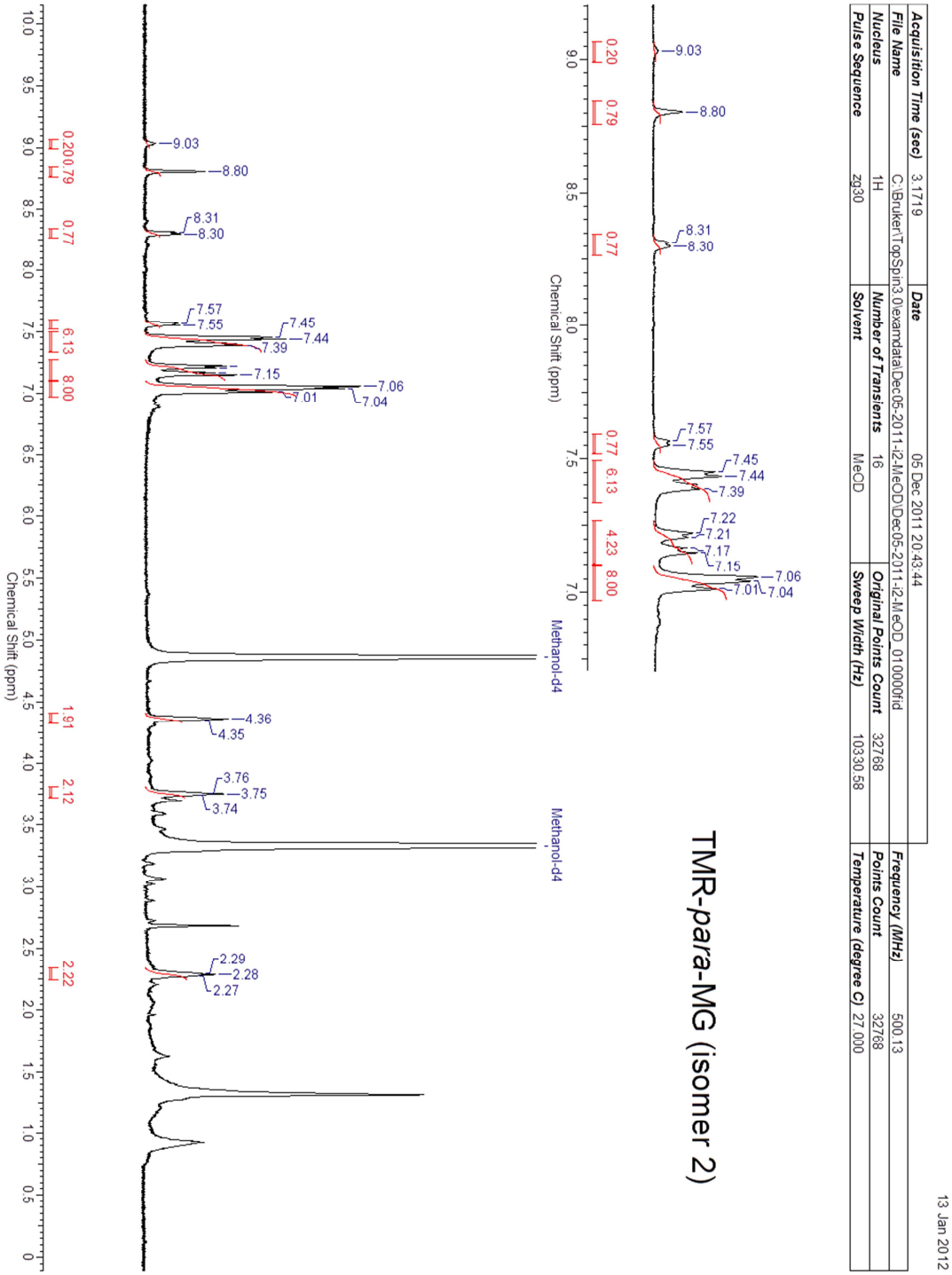
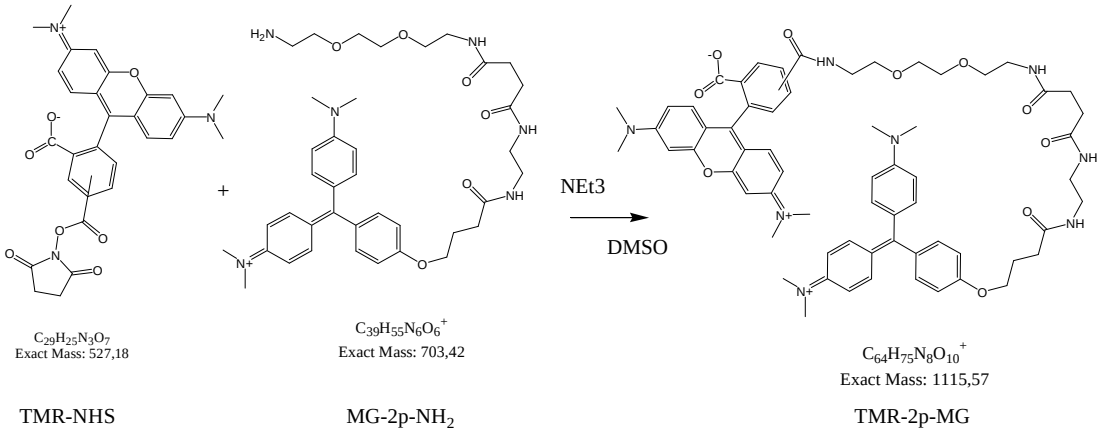


Figure 5. ^{1}H NMR (500 MHz in MeOD) spectrum of TMR-*para*-MG dye (single purified)

TMR-2p-*meta*-MG and TMR-2p-*para*-MG

TMR-2p-MG dyes were synthesized according to scheme 2 from MG-2p-NH_2 [1] and TMR-NHS.



Scheme 2. Scheme of synthesis of TMR-2p(*meta*)-MG and TMR-2p*(para*)-MG dyes.

Protocol:

In a dry (5 ml) round flask, flushed with Ar and containing a stirrer bar, MG-2p-NH_2 (15 mg, 22.7 µmol) was dissolved in dry DMSO (0.5 ml). Then NEt_3 (40 µl , 300 µmol) and TMR-NHS (9 mg, 17.0 µmol), mixture of two isomers, in DMSO (0.5 ml) was added. The reaction mixture was left stirring overnight at room temperature. Next day an aliquot (1 µl) for HPLC and an aliquot (1 µl) for mass spec were taken in order to check formation of TMR-2p-MG (Scheme 2).

Both isomers have very similar retention time on HPLC and first were purified as mixture of two isomers.

They were then separated by prep HPLC applying the following gradient (A = H_2O, 0.1%TFA and B = CH3CN): A/B 90:10 for 5 min, to A/B 70:30 in 15 min and to 45:55 in 55 min.

Isomer 1 was attributed to *meta* isomer and 2 to *para* isomer according to NMR spectra similarly to MG-*meta/para*-TMR.

TMR-2p*(meta*)-MG (Isomer 1): ^{1}H NMR (MeOD, 500 MHz, ppm), δ = 2.15 (t, *J* = 7.0, 2H), 2.45 (m, 6H), 3.49 (t, *J* = 5.4, 2H), 3.61 (m, 8H), 4.19 (t, *J* =6.4, 2H), 6.99 (s, 2H), 7.06 (m, 6H), 7.16 (m, 4H), 7.35 (d, *J* = 8.5, 2H), 7.42 (d, *J* = 8.5, 4H), 7.84 (s, 1H), 8.23 (d, *J* = 7.9, 1H), 8.42 (d, *J* = 8.1, 1H), 8.79 (bs, 1H); m/z = 1115,6

TMR-2p*(para*)-MG (Isomer 2): ^{1}H NMR (MeOD, 500 MHz, ppm), δ = 2.15 (t, *J* = 7.1, 2H), 2.45 (m, 6H), 3.57 (t, *J* = 5.3, 2H), 3.67 (m, 8H), 4.19 (t, *J* =6.3, 2H), 6.99 (s, 2H), 7.06 (m, 6H), 7.16 (m, 4H), 7.37 (d, *J* = 8.6, 2H), 7.42 (d, *J* = 7.9, 4H), 7.53 (d, *J* = 7.9 , 1H), 8.28 (d, *J* = 7.9 ,1H), 8.79 (s, 1H); m/z = 1115,6

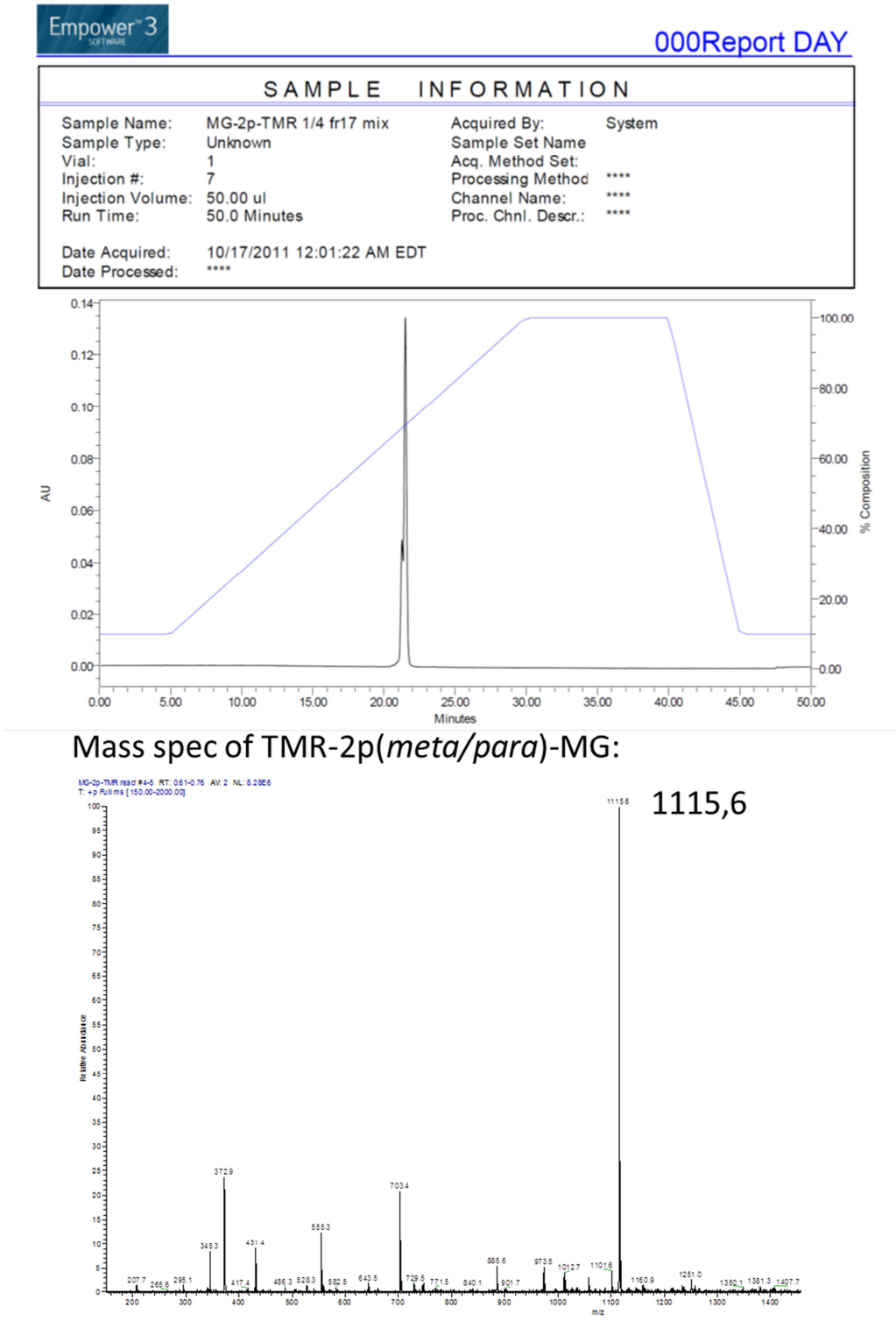


Figure 6. HPLC chromatogram and mass spectrum of TMR-2p(*meta/para)*-MG dyes

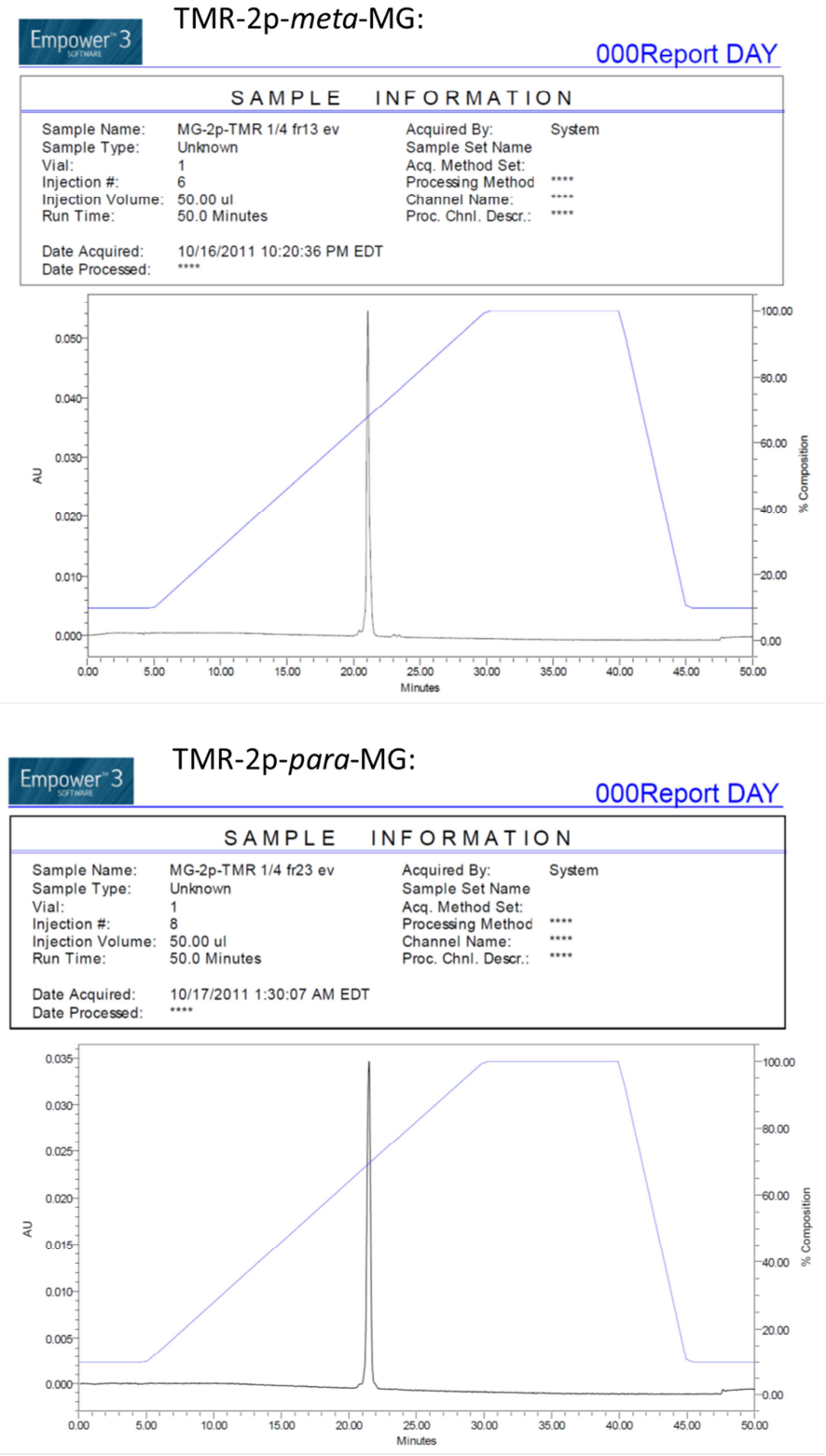


Figure 7. Analytical HPLC chromatograms of TMR-2p(*meta/para)*-MG dyes after prep HPLC separation (double purified)

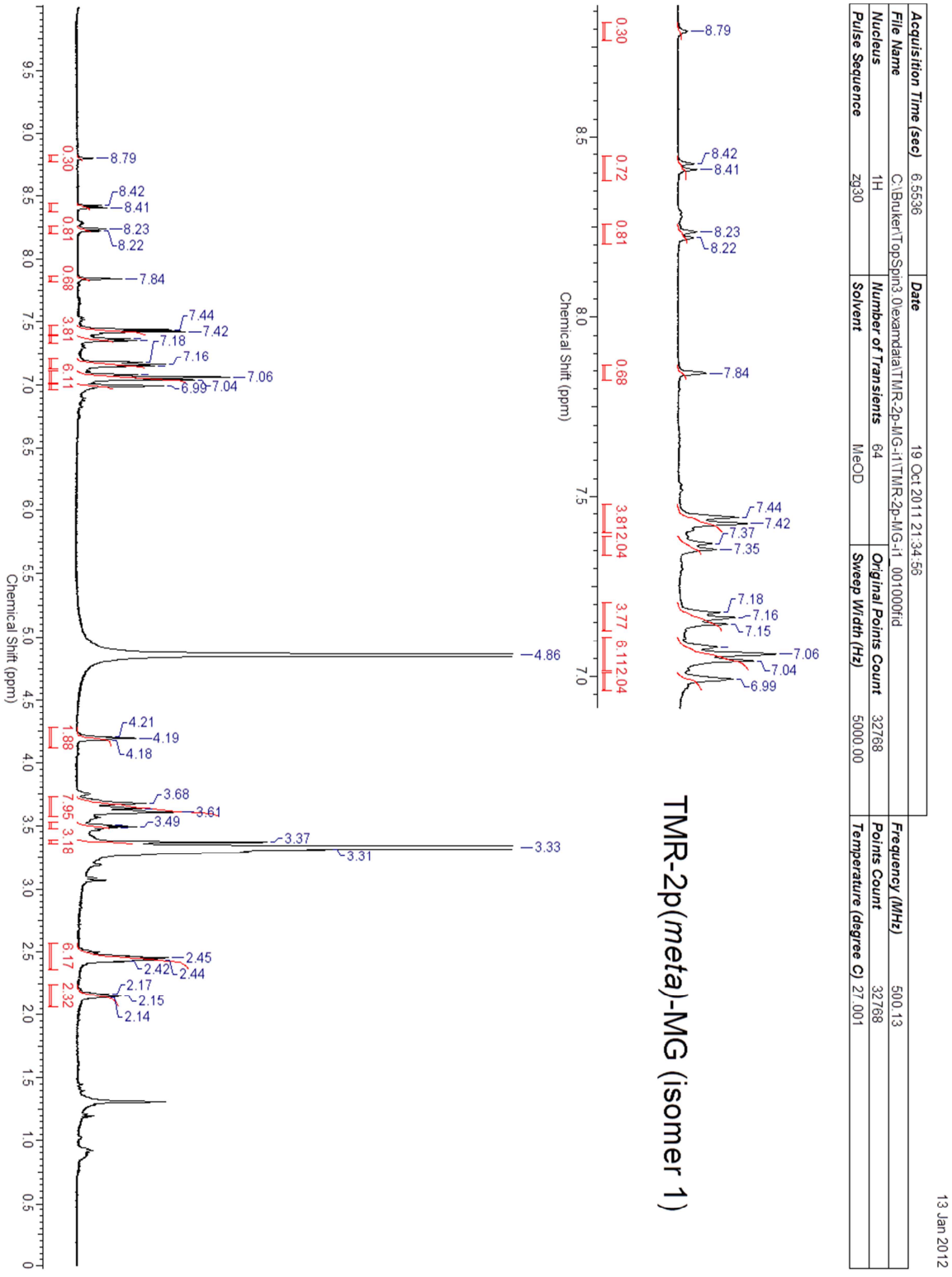


Figure 8. ^{1}H NMR (500 MHz in MeOD) spectrum of TMR-2p(*meta*)-MG dye (single purified)

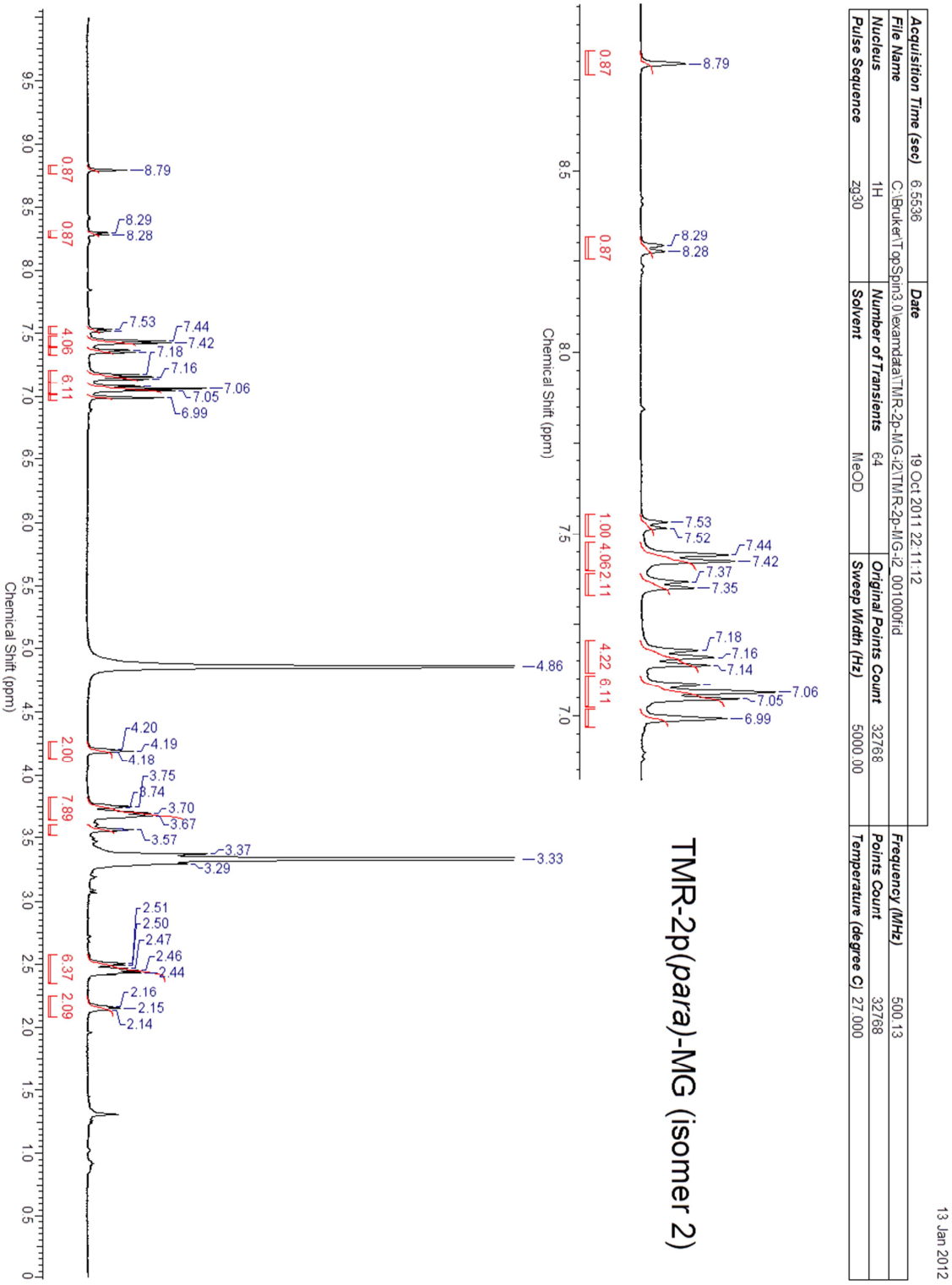


Figure 9. ^{1}H NMR (500 MHz in MeOD) spectrum of TMR-2p(*para)*-MG dye (single purified)

TMR is a suitable FRET donor for MG dye

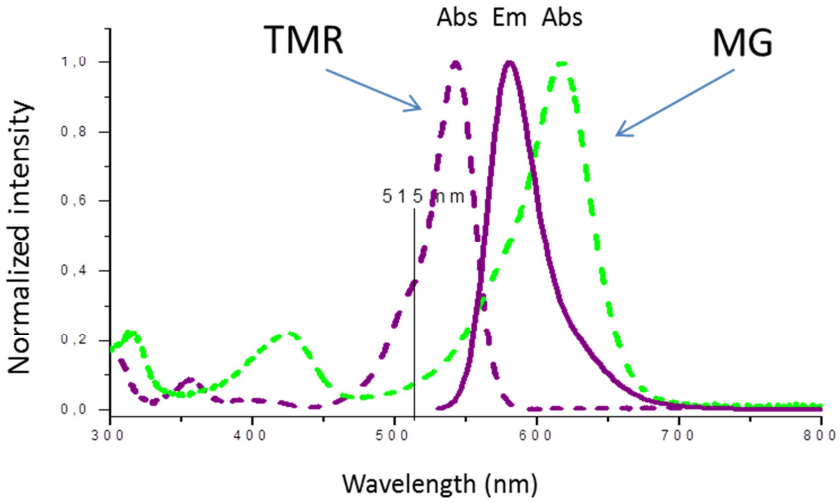


Figure 10. Normalized absorption and emission spectra of TMR and MG chromophores in PBS buffer. As TMR chromophore a methyl ester of tetramethylrhodamine (Invitrogen, #T668) was used, as MG chromophore MG-NH_2 was used.

Spectroscopic properties of TMR-MG dyes

Table 1. Spectroscopic properties of TMR, MG and TMR-MG dyes

Dye	Environment	Absorption, nm		Emission, nm	QY
		λmax_1	λmax_2	λmax	
TMR-OMe	EtOH + 5% AcOH	551	-	574	41.3
	PBS^+, pH = 7.4	552	-	576	28.7
	PBS^+, pH = 7.4 + FAP	553	-	578	29.4
MG-NH_2	EtOH + 5% AcOH	-	609	-	<<0.1
	PBS^+, pH = 7.4	-	609	-	<<0.1
	PBS^+, pH = 7.4 + FAP	-	635	658	12.5
TMR-meta-MG	EtOH + 5% AcOH	553	609	574	0.88
	PBS^+, pH = 7.4	535	635	578	0.25
	PBS^+, pH = 7.4 + FAP	556	635	660	6.5
TMR-para-MG	EtOH + 5% AcOH	553	609	576	0.98
	PBS^+, pH = 7.4	553	611	580	7.0
	PBS^+, pH = 7.4 + FAP	556	634	658	13.5
TMR-2p(meta)-MG	EtOH + 5% AcOH	553	609	576	1.8
	PBS^+, pH = 7.4	543	622	580	1.5
	PBS^+, pH = 7.4 + FAP	558	639	662	8.6
TMR-2p(para)-MG	EtOH + 5% AcOH	553	608	576	1.6
	PBS^+, pH = 7.4	542	622	578	1.4
	PBS^+, pH = 7.4 + FAP	560	642	664	8.4

Comparison of absorbance spectra of TMR-MG dyes

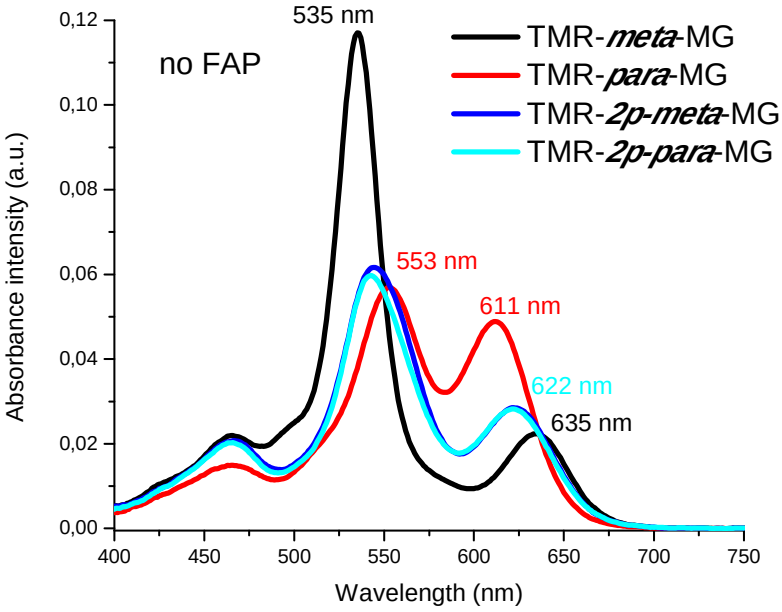


Figure 11. Comparison of the absorbance properties of TMR-MG dyes in PBS^{+}, pH=7.4: TMR-2p-MG dyes have very similar absorbance while TMR-*meta*-MG and TMR-*para*-MG are very different

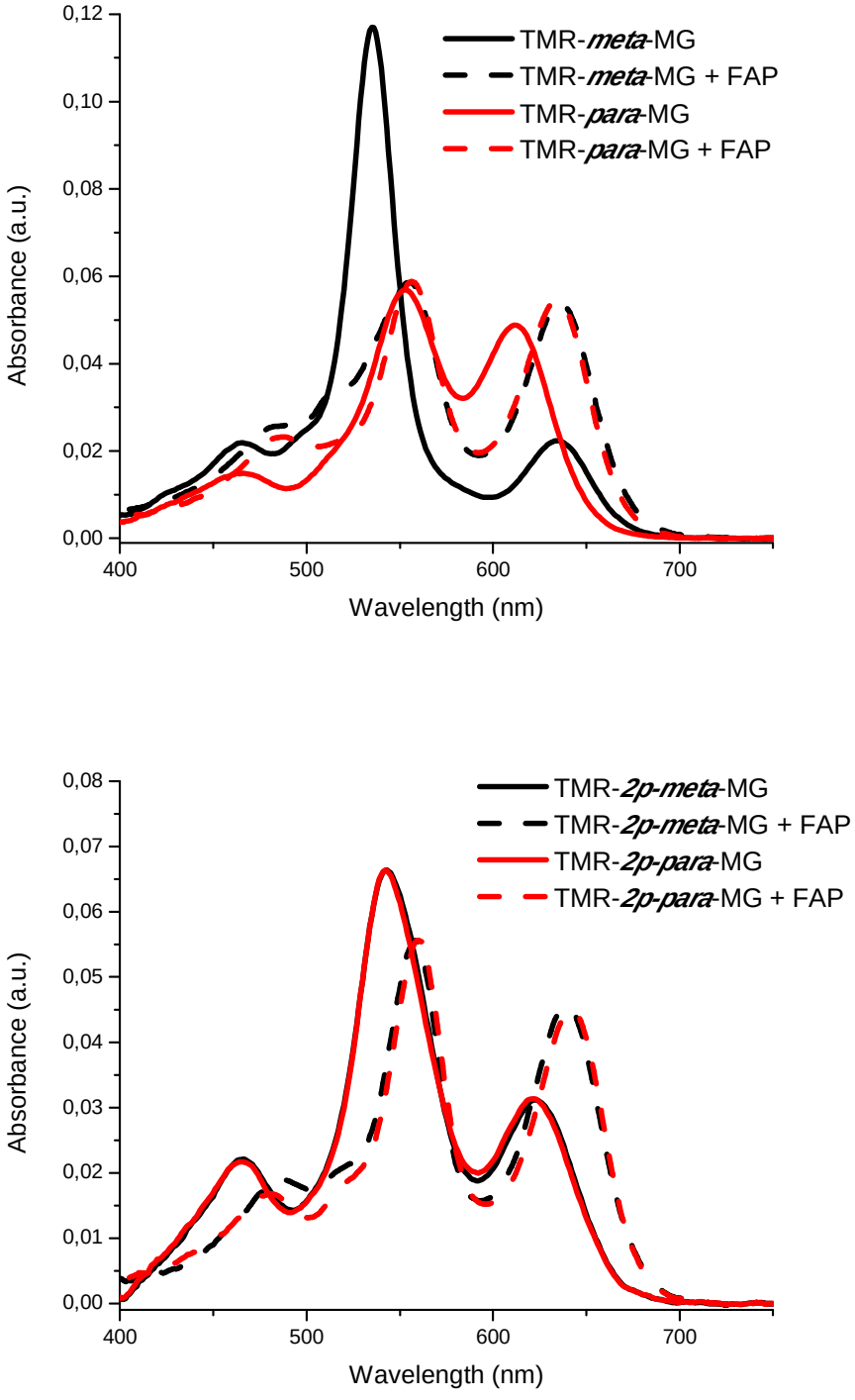


Figure 12. Comparison of the absorbance properties of TMR-MG dyes in PBS^{+}, pH=7.4 in the absence and presence of FAP.

Absorbance spectra of TMR-MG dyes: EtOH vs buffer

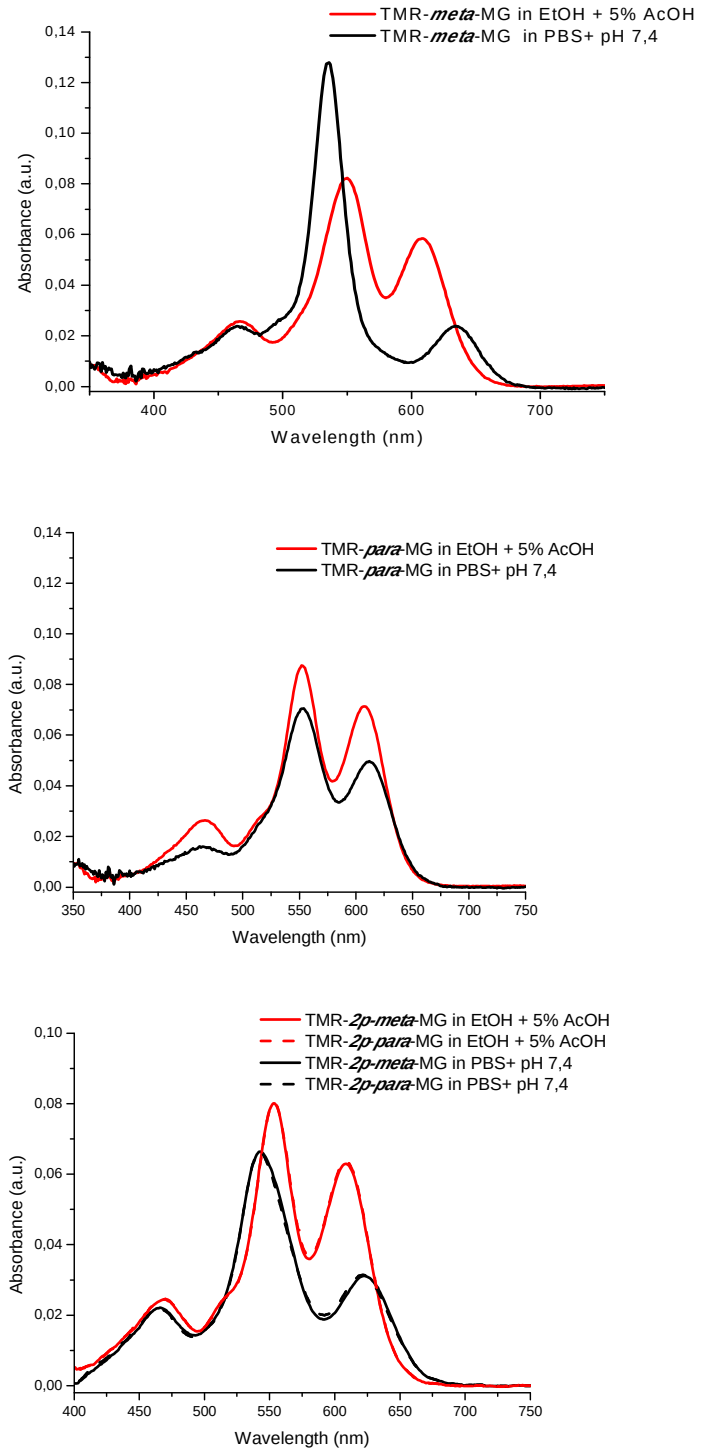


Figure 13. Comparison of the absorbance properties of TMR-MG dyes in PBS^{+}, pH=7.4 and in EtOH (+5%AcOH).

Fluorescent spectra of TMR-2p-MG dyes

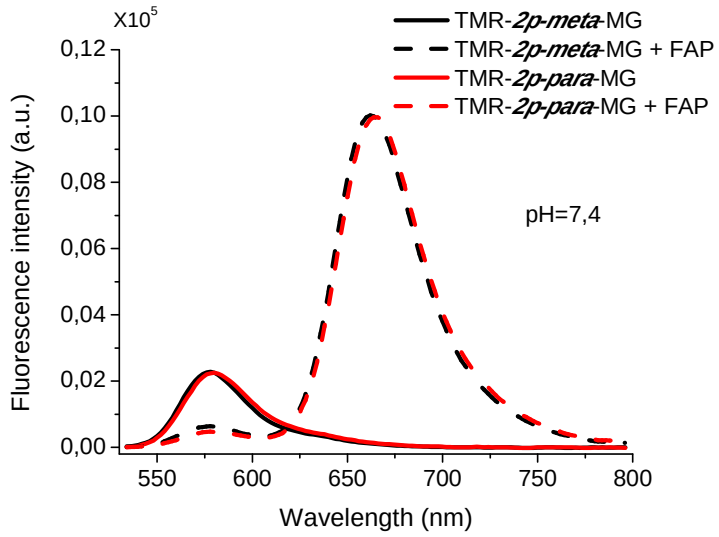


Figure 14. Fluorescence spectra of TMR-2p-MG dyes in PBS^+, pH=7.4 in the absence and presence of FAP.

MG is a pH-sensitive dye

The MG dye exists in equilibrium between two forms at physiological pHs: the dye form which is characterized by strong absorbance with maximum at ~610 nm and the decolourized carbinol form:

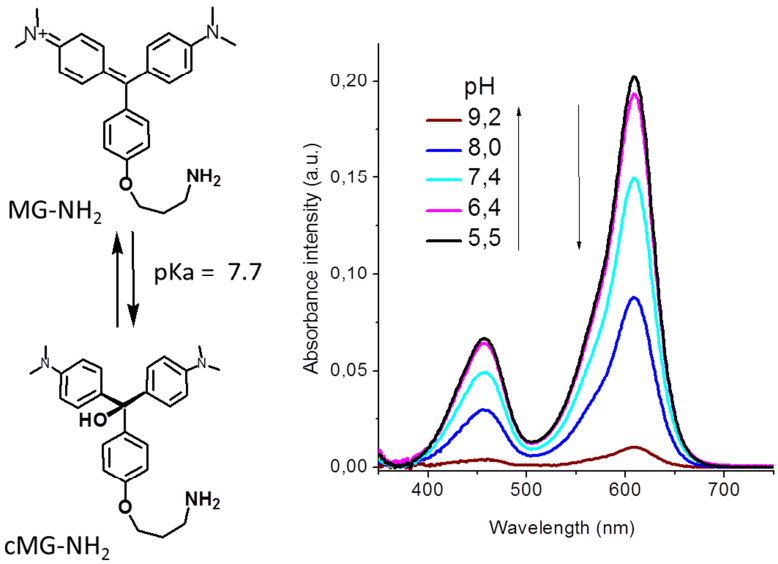


Figure 15. Schematic representation of the pH dependent equilibrium between dye and carbinol forms for $MG\text{-}NH_2$ dye and its absorbance spectra as function of pH

pKa of MG-NH_2 and TMR-MG:

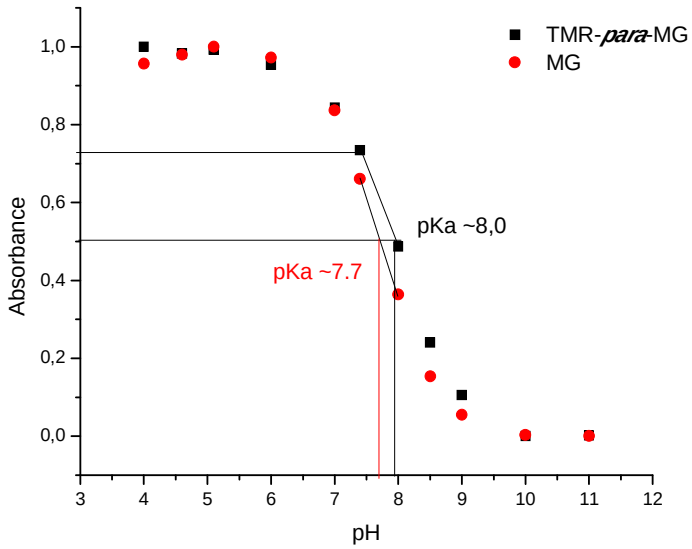


Figure 16. Determination of pKas for MG and TMR-*para*-MG dyes (in PBS^+, pH=7.4).

Kinetics of TMR-MG binding to FAP

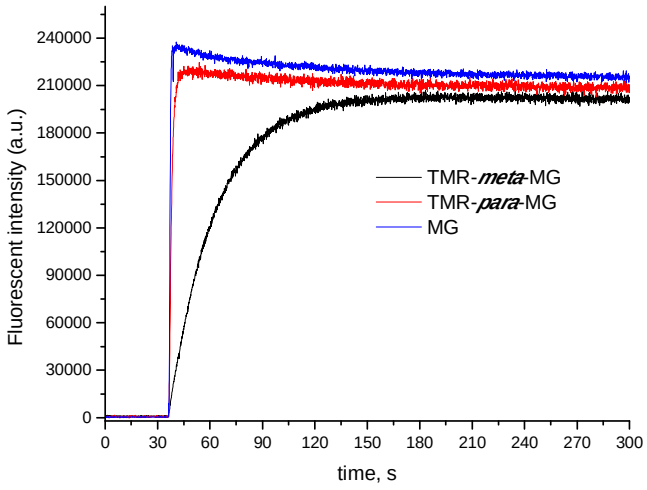


Figure 17. Comparison of binding kinetics of MG and TMR-MG dyes with FAP. Concentration of dyes is 0.4uM, of FAP is 1.1uM. While TMR-*para*-MG shows binding kinetics very similar to MG-NH_2, TMR-*meta*-MG binds to FAP much more slowly, probably due to the relatively slow dissociation of the intramolecular heterochromophore complex.

Concentration dependence of TMR-*meta*-MG absorbance

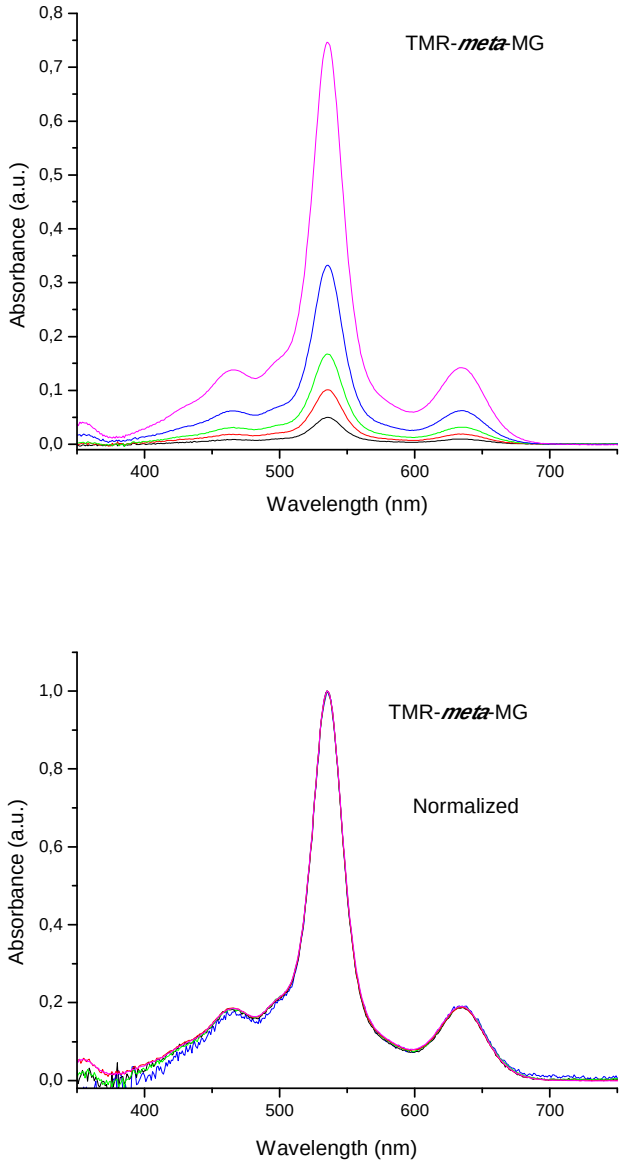
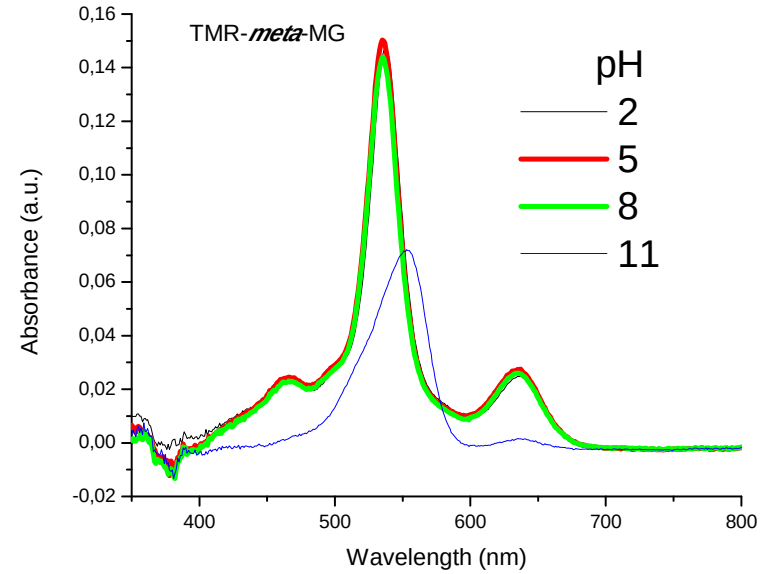


Figure 18. Comparison of absorbance spectra of TMR-*meta*-MG dye in PBS^{+}, pH=7.4 at various concentrations. The fact that the shape of the absorbance spectrum does not change with concentration supports the hypothesis of intramolecular heterochromophore complex formation.

TMR-*meta*-MG dye is sensitive to pH only in very basic environment



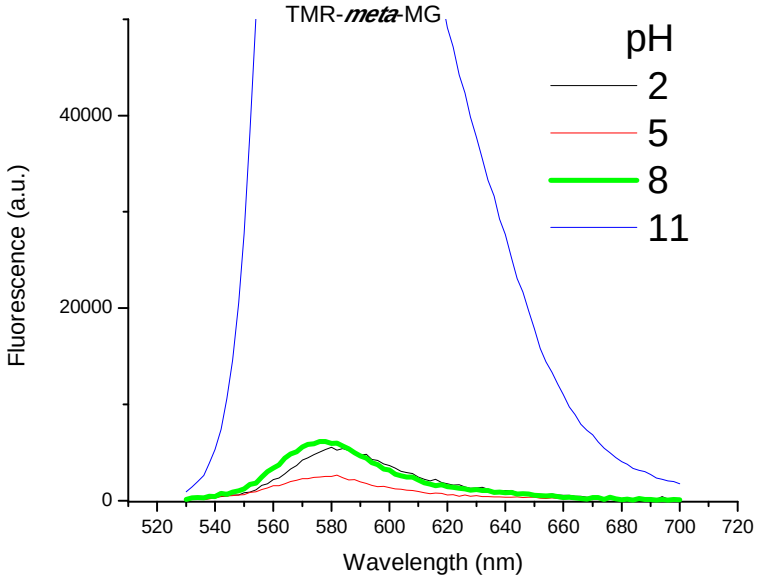


Figure 19. Absorbance and fluorescence spectra of TMR-*meta*-MG dye in PBS^+ at different pH.

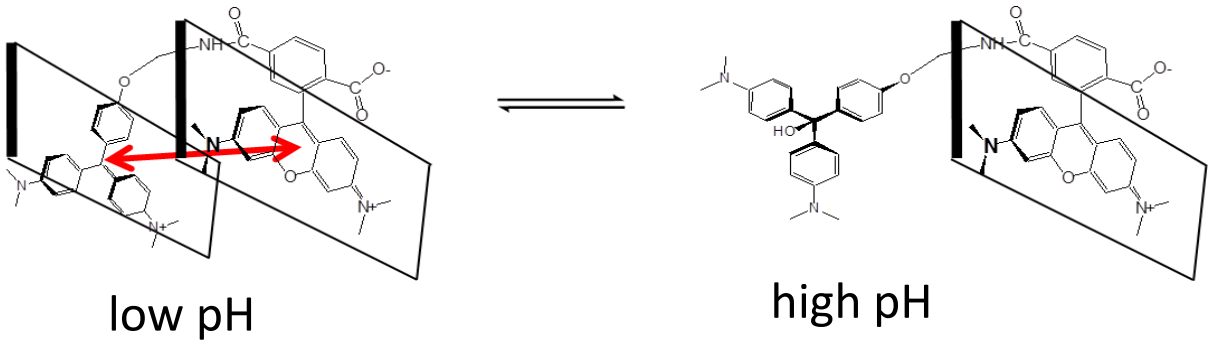
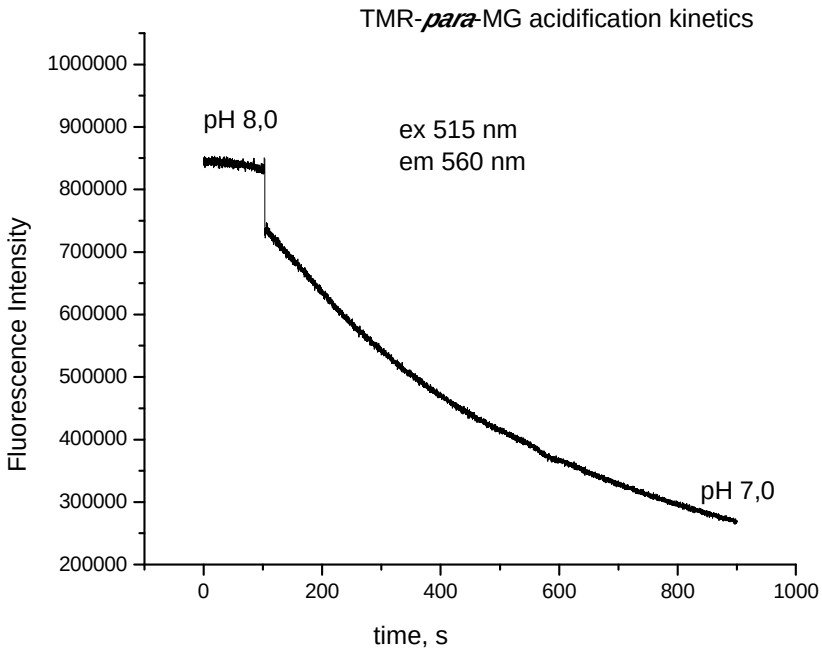


Figure 20. Hypothetical scheme of the chromophore - chromophore interaction in TMR-*meta*-MG dye at different pHs.

pH response of TMR-para-MG fluorescence



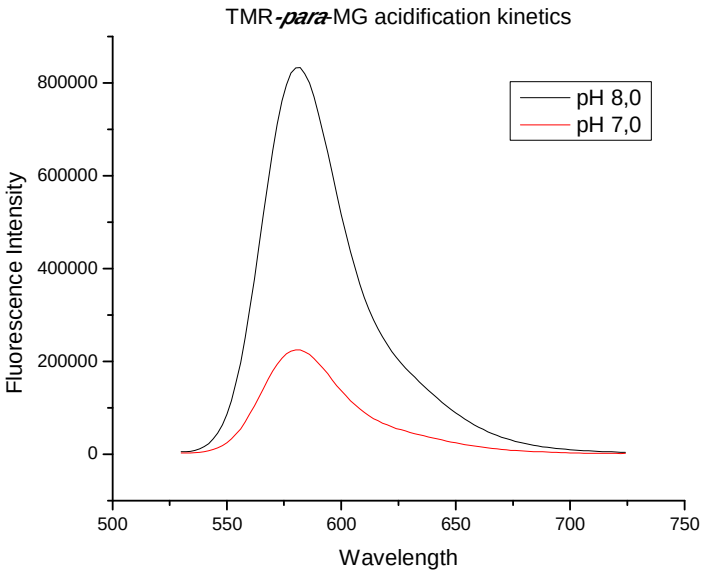
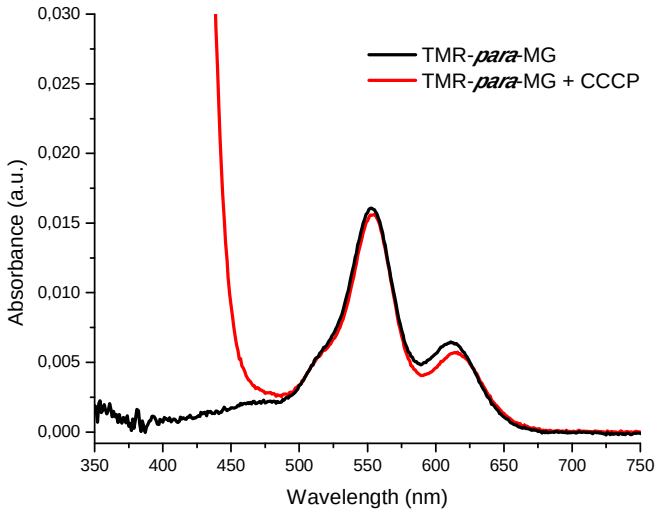


Figure 21. TMR-*para*-MG acidification kinetics. The dye was excited at 515 nm and emission was collected at 560 nm. TMR-*para*-MG is a slow-responding pH sensitive dye.

Effect of CCCP on TMR-*para*-MG fluorescence



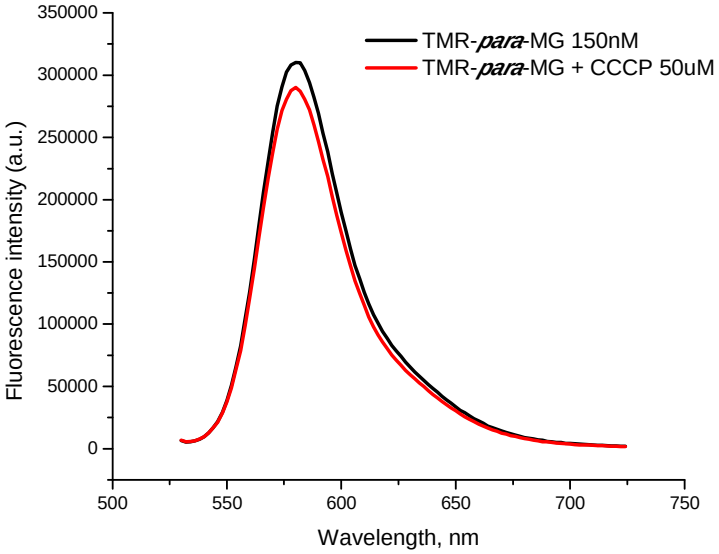


Figure 22. Absorbance and emission spectra of TMR-*para*-MG dye (150 nM) in the absence and presence of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 50uM), a chemical inhibitor of oxidative phosphorylation and a drug causing depolarization of mitochondria in living cells

Cells that express FAPs

HEK 293_B7-1_dL5

Construction of pOI constructs for surface expression of FAPs in mammalian cells.

The design of the transmembrane anchor was adapted from previously described methods (Chou, et al., 1999): primer overlap PCR was used to assemble primers pOI01 – pOI06, generating a PCR amplicon that encodes an Igκ signal peptide, an HA epitope, a XbaI/XmnI cloning site immediately preceding murine B7-1 from Pro237 to the terminal leucine, and a SpeI/XmaI cloning site. The amplicon was gel purified and digested by NheI and XmaI restriction enzymes. The plasmid pEGFP-C2-Rif, a kind gift from the lab of Dr. Manojkumar Puthenveedu, was similarly digested and gel purified to obtain a vector backbone with compatible terminal overhangs. The digested amplicon and digested vector backbone were ligated in a 1:3 molar excess of amplicon to generate *pOI* and transformed into chemically competent *E. coli* using standard heat-shock methods.

Construction of pOIdL5C.

The gene encoding dL5(E52D L91S) was flanked by XbaI and XmaI restriction sites via PCR amplification using primers pOIdL5CFw and pOIdL5CRv. The PCR amplicon was gel purified, digested with XbaI and

XmaI, and ligated into like-digested pOI to generate *pOIdL5C*, which was then transformed into chemically competent *E. coli* by standard heat-shock methods.

Transfection of HEK293 cells using pOIdL5C.

HEK293 cells were plated in a 6-well tissue culture tray and grown to approximately 80% confluence in DMEM supplemented with 10% bovine serum. The cells were then transfected with pOIdL5C (1 µg/µL stock) using Fugene 6 (Roche Applied Science) in accordance with manufacturer-provided protocols. Transfected cells were selected by growth in media supplemented with G418 (500 µg/mL). Transfected cells were sorted by their ability to activate fluorescence in malachite green ester (MGE, 100 nM) using a Becton Dickinson FACSVantage SE with FACSDiva option (633 nm excitation: 685/35 emission). Cells exhibiting high fluorescence intensity were cloned by sorting single cells into each well of a 96-well tissue culture tray. Clones were propagated in DMEM with 10% bovine serum and G418 (500 µg/mL), and FAP activity was verified by confocal microscopy prior to harvesting and preservation of these stable cell lines in liquid N_2.

Primer Name	Sequence
pOI01	agatccgctagcgctaccggtcgccaccATGgagacagacacactcctgctatgggtactgctgctctgggttcc
pOI02	tcctctagaagcataatctggaacatcatatggatagtcaccagtggaacctggaacccagagcagcagtacc
pOI03	ccatatgatgttccagattatgcttctagaggaggaggtgcgaagaagttccccccagaagaccctcctgatagcaagaacacacttgtgct ctttgg
pOI04	gtgcttacagaagcatttgatgatgacaacgatgacgacgactgttattactgcgccgaatcctgccccaaagagcacaagtgtgttcttgc tatc
pOI05	gttgtcatcatcaaatgcttctgtaagcacagaagctgtttcagaagaaatgaggcaagcagagaaacaaacaacagccttaccttcg
pOI06	ctattaCCCGGGctattaactagtaaggaagacggtctgttcagctaatgcttcttcaggcccgaaggtaaggctgttgtttgtttctct
pOIdL5CFw	aaggggtaactagtgaaaacctgtacttccagggctctcagg
pOIdL5CRv	ctattacccgggtccaccgcctccggagagg

Hela_dH6

The PCR-amplified dH6 FAP fragments were first cloned into pAcGFP1-Actin (Clontech, Mountain View, CA) to replace GFP using NheI and BglII and a FAP-Actin fusion gene was generated. The FAP-Actin fusion gene was cut out using AgeI and BamHI and ligated into AgeI/BamHI-digested retroviral vector pQCXIP (Clontech, Mountain View, CA). Retrovirus packaging and subsequent infection of Hela cells were performed according to the manufacturer's instructions. Infected Hela cells were selected using 600 ng/ml puromycin and stable cells were maintained with 200 ng/ml puromycin.

Yeasts_dL5

Yeast cells were grown in SGR+CAA medium to induce expression of surface displayed dL5(E52D L91S) FAP similarly to the protocol described in [3]. Cells were then directly affixed to a concanavalin A coated Mattek dish and overlain with 1 ml of the same medium containing 150 nM of corresponding dye.

Confocal microscopy: comparison of TMR-*meta*-MG and TMR-*para*-MG dyes

Some representative examples of confocal images of FAP expressing HEK cells stained with 150 nM TMR-MG dyes

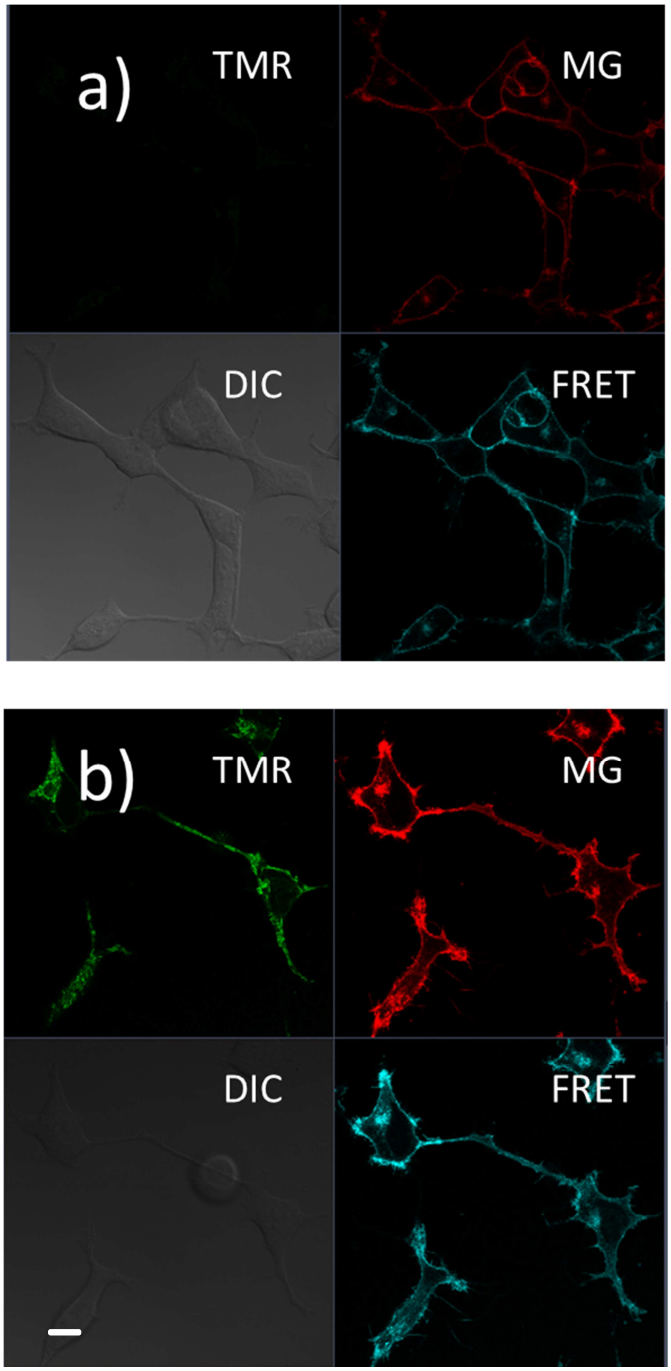


Figure 23. Confocal images of a) TMR-*meta*-MG and b) TMR-*para*-MG dyes. A set of setting used to acquire the images is shown in table 2 (see below).

Table 2. Typical settings for Carl Zeiss LSM-510_META_MP_ConfoCor 3 Confocal Microscope

Scaling X and Y	0.14 µM
Image size	1024x1024, 16-bit
Dimensions	142,72x142,72 µm
Objective	63x/1.4 Oil DIC
Pixel dwell	1.6 µs
Average	4
Master Gain	561TMR: 720 633MG: 760 DIC: 320 FRET: 760
Pinhole	561TMR: 110 µm 633MG: 124 µm FRET: 124 µm
Filters	561TMR: BP 575-630 IR 633MG: BP 650-710 IR FRET: BP 650-710 IR
Beam splitters	MBS : HFT 405/488/561/633/KP 725 DBS1 : Mirror DBS2 : NFT 565 FCS EF : BG 39 FCS DBS : NFT 490
Lasers	561TMR: 561 nm : 1.0 % 633MG: 633 nm : 6.0 % FRET: 561 nm : 1.5 %
In case of 2-photon excitation 850 nm excitation laser was used, 3%	

Colocalization of TMR-*para*-MG dye with MitoTracker

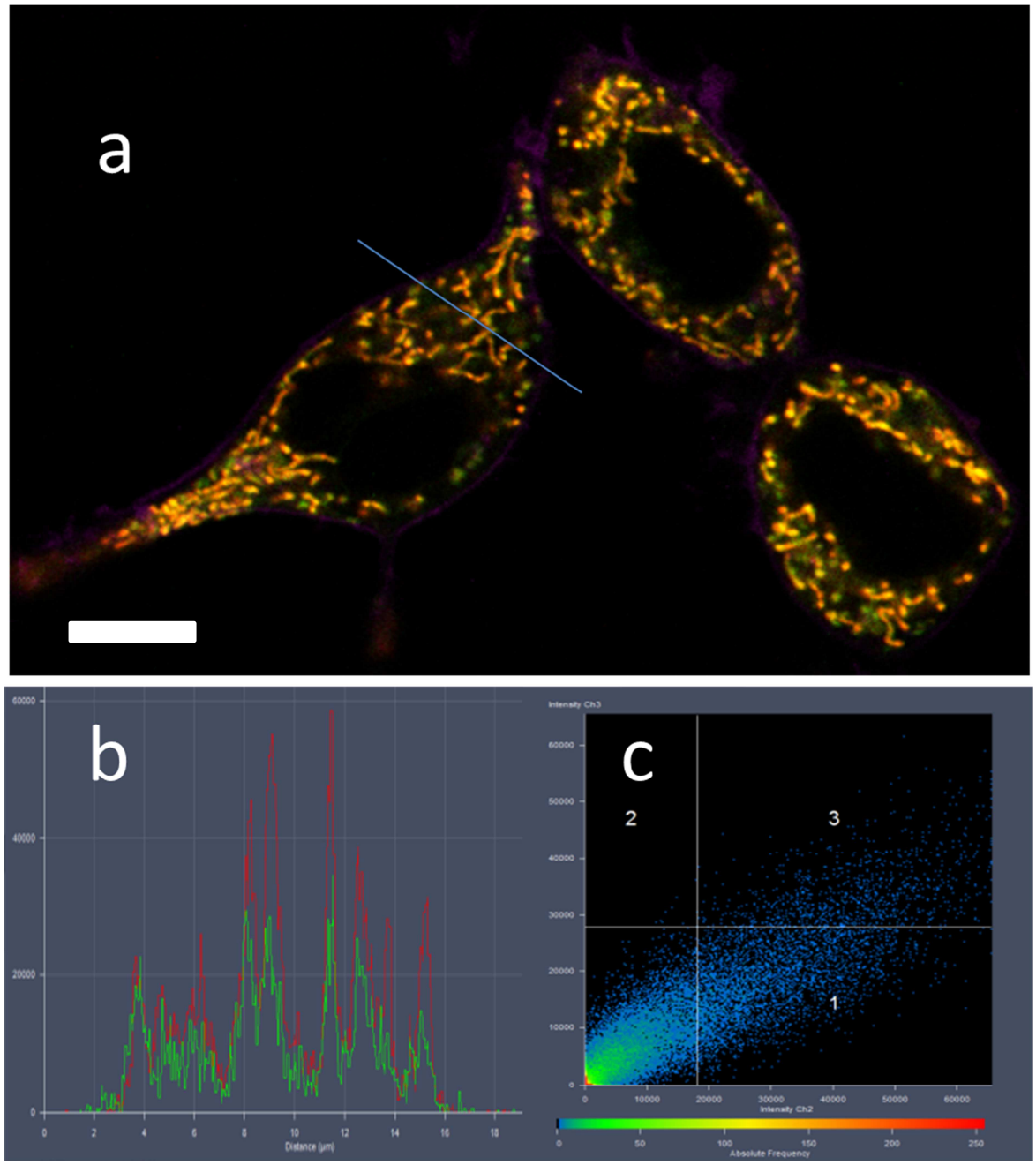


Figure 24. Colocalization of TMR-*para*-MG and MitoTracker Green (Invitrogen, #M-7514): a) Confocal image of HEK cells labeled with TMR-*para*-MG (green free dye, pink – FAP bound dye) and MitoTracker (red) which was added after TMR-*para*-MG; b) Intensity profiles of TMR-*para*-MG (green) and MitoTracker (red) that correspond to the line on the panel (a); c) Plot of TMR-*para*-MG signal versus signal of MitoTracker. Concentrations of dyes were: TMR-*para*-MG 150nM and MitoTracker Green 100nM. Scale bar is 10 µm

Mitochondria depolarization of the cells labeled with TMR-*para*-MG as a result of their treatment with FCCP

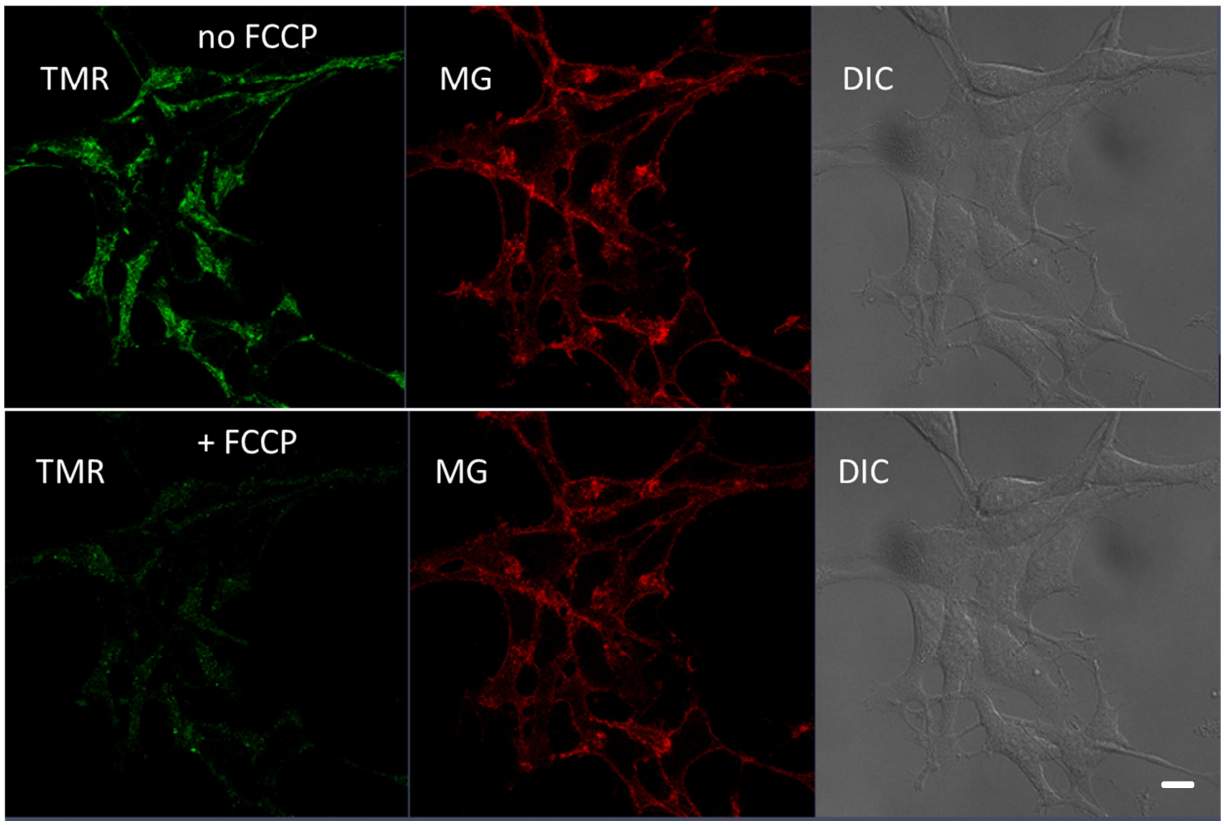


Figure 25. Comparison of confocal microscope images of the HEK 293 cells that express dL5 FAP fused to the transmembrane domain of B7-1 protein in the presence of 150nM TMR-*para*-MG before and after treatment (after 20s of treatment) with 40µM FCCP (Carbonyl cyanide 4-trifluoromethoxyphenylhydrazone). Scale bar is 10 µm.

Mitochondria depolarization of the cells labeled with TMRM as a result of their treatment with CCCP

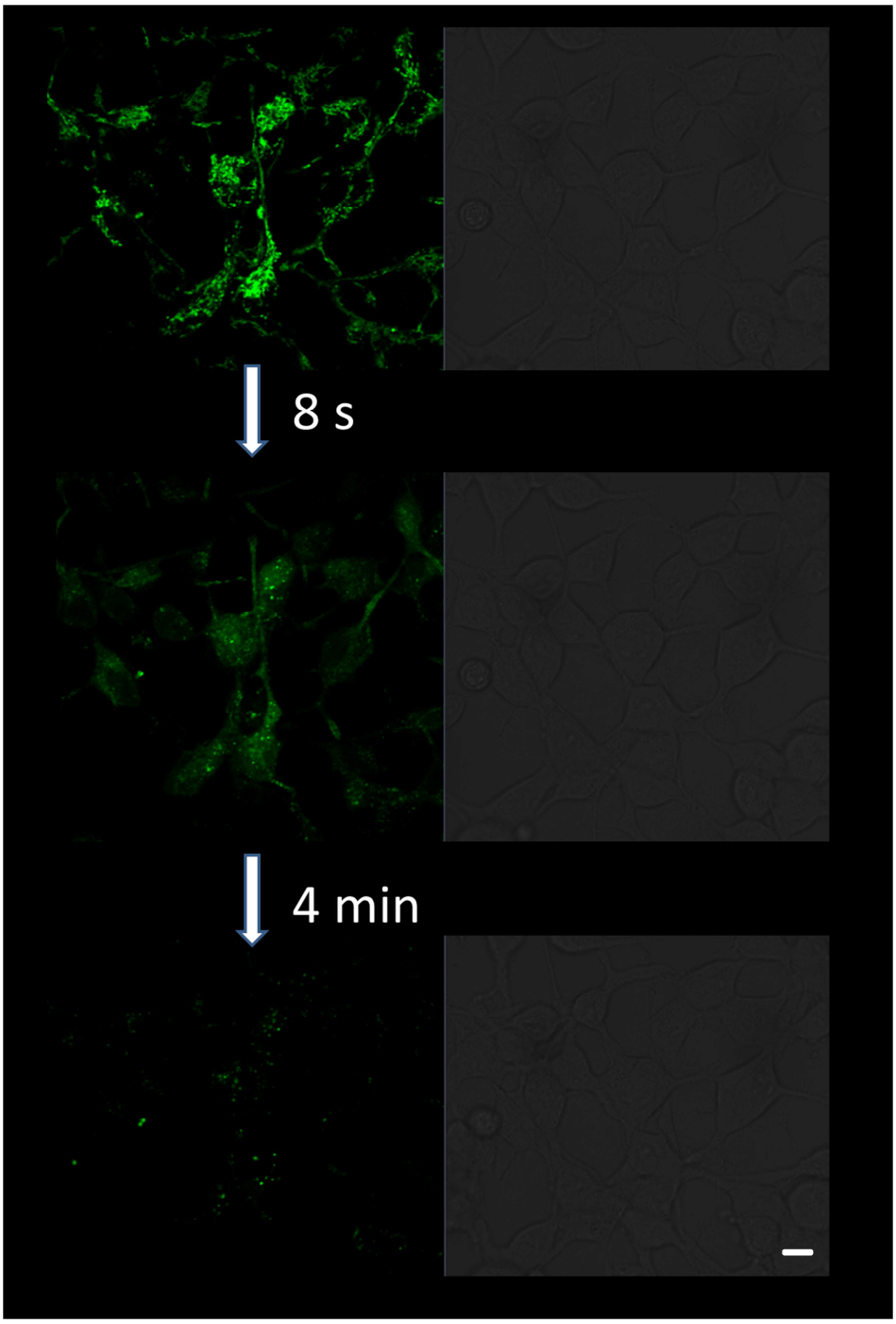


Figure 26. Time series of confocal microscope images of the HEK 293 cells that express dL5 FAP fused to the transmembrane domain of B7-1 protein in the presence of 50nM TMRM dye after treatment with 100µM CCCP. Scale bar is 10 µm.

NMR characterization of TMR-MG tandem dyes

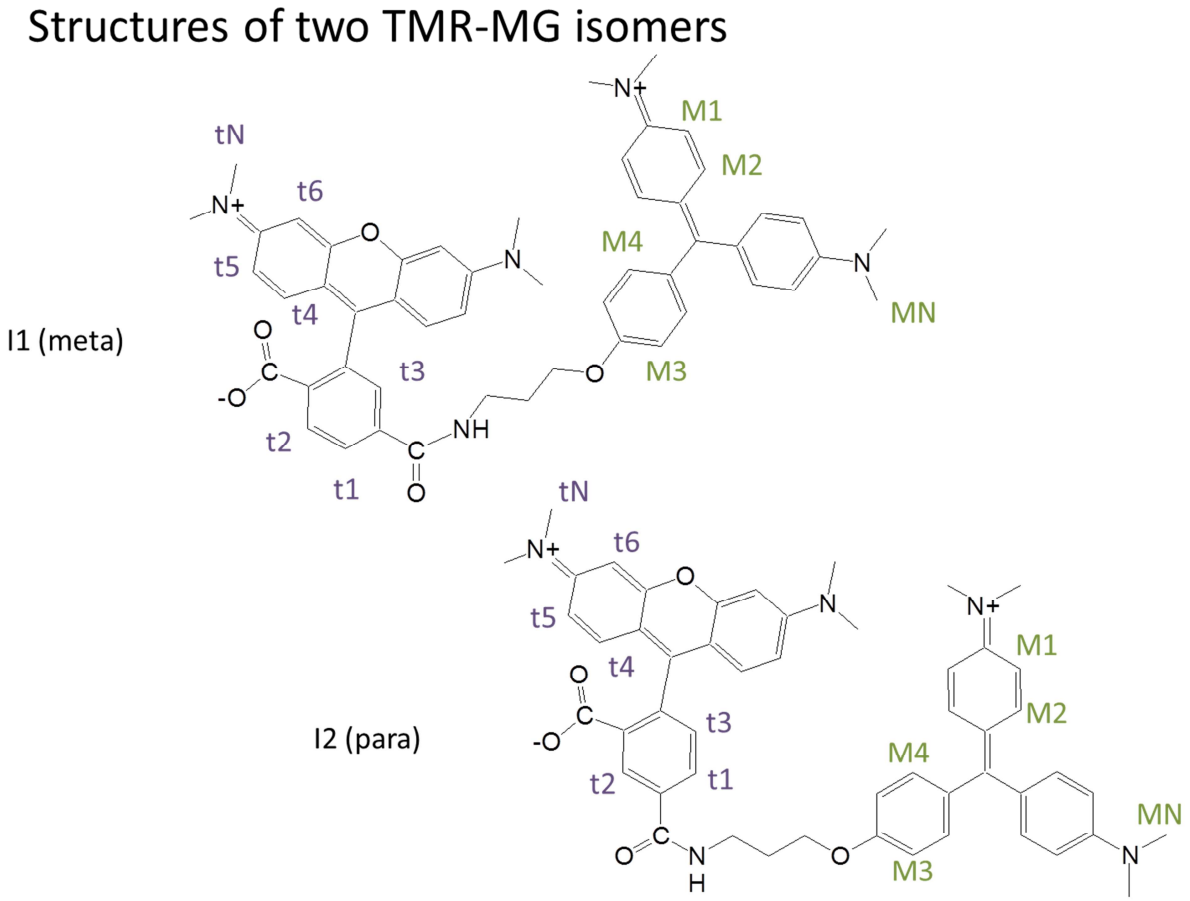
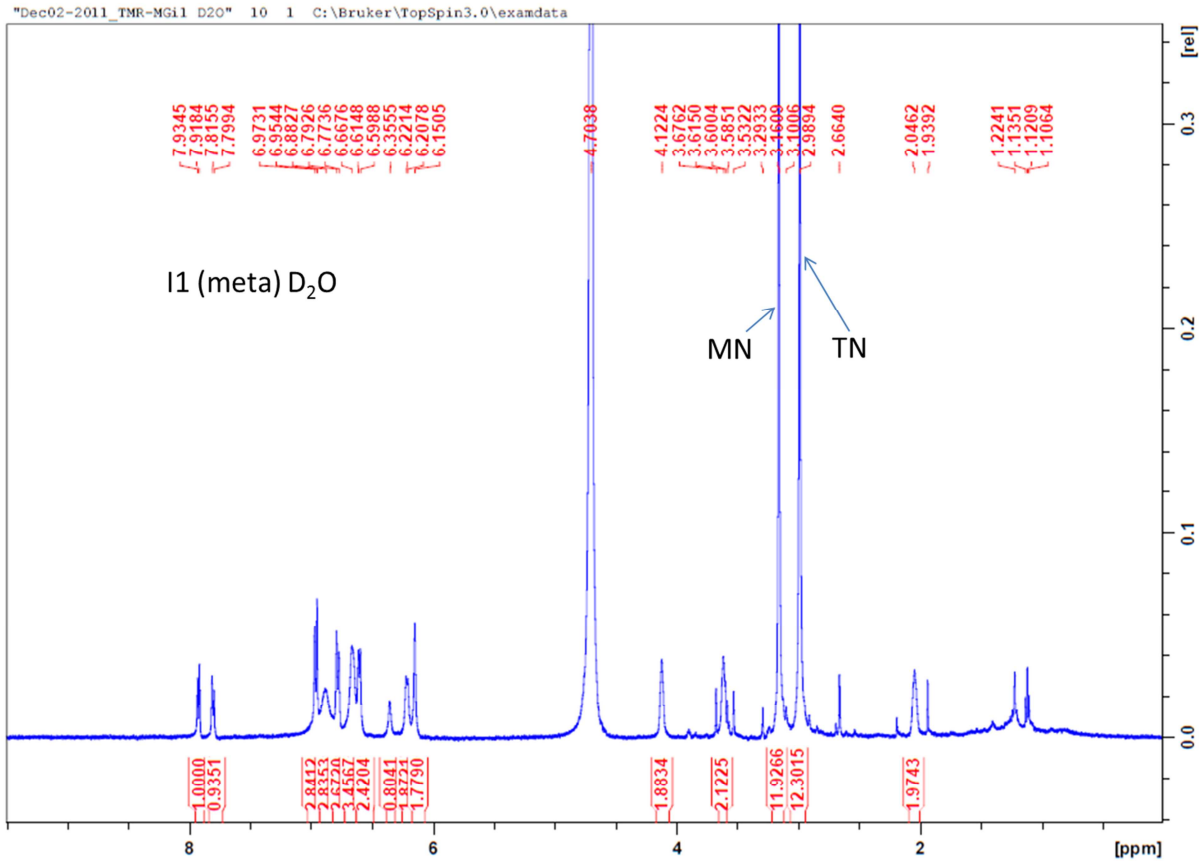
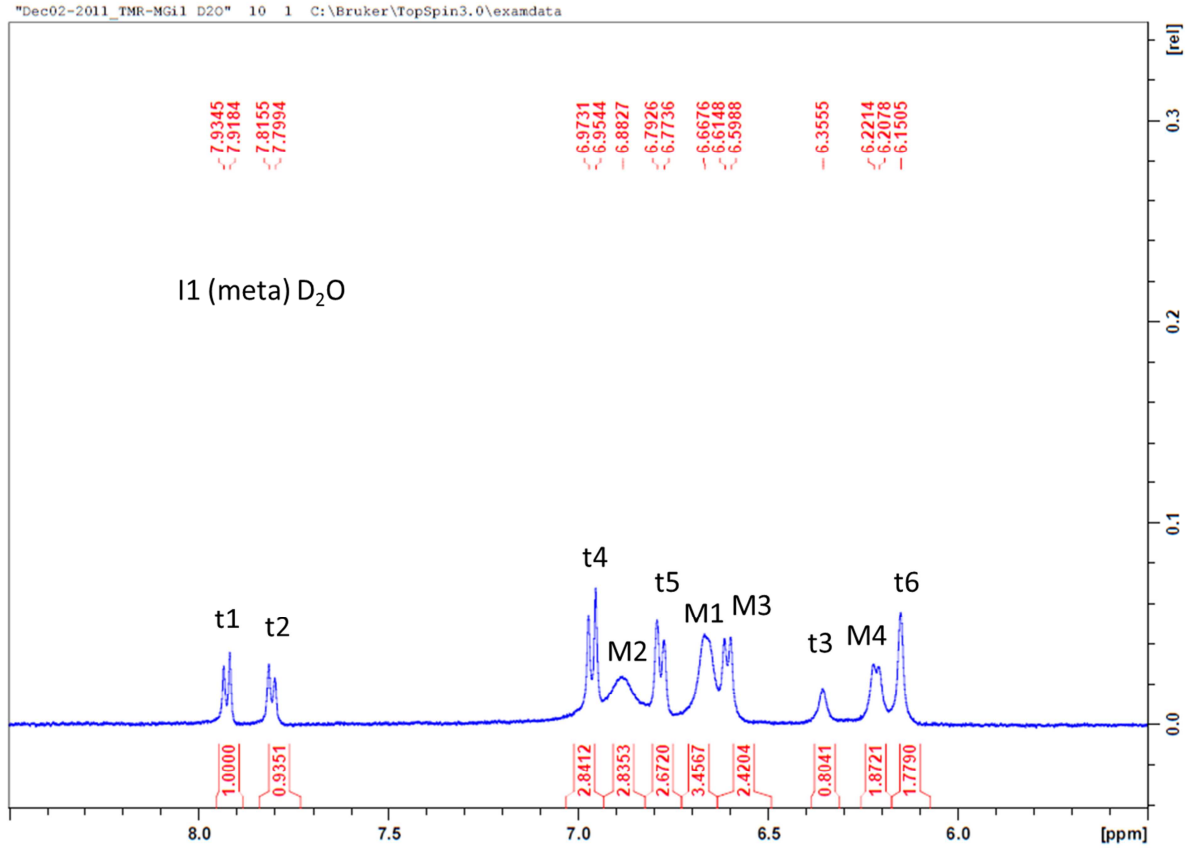


Figure 27. Structures of both TMR-MG isomers with numeration used for the signals assignment in their NMR spectra (see below).

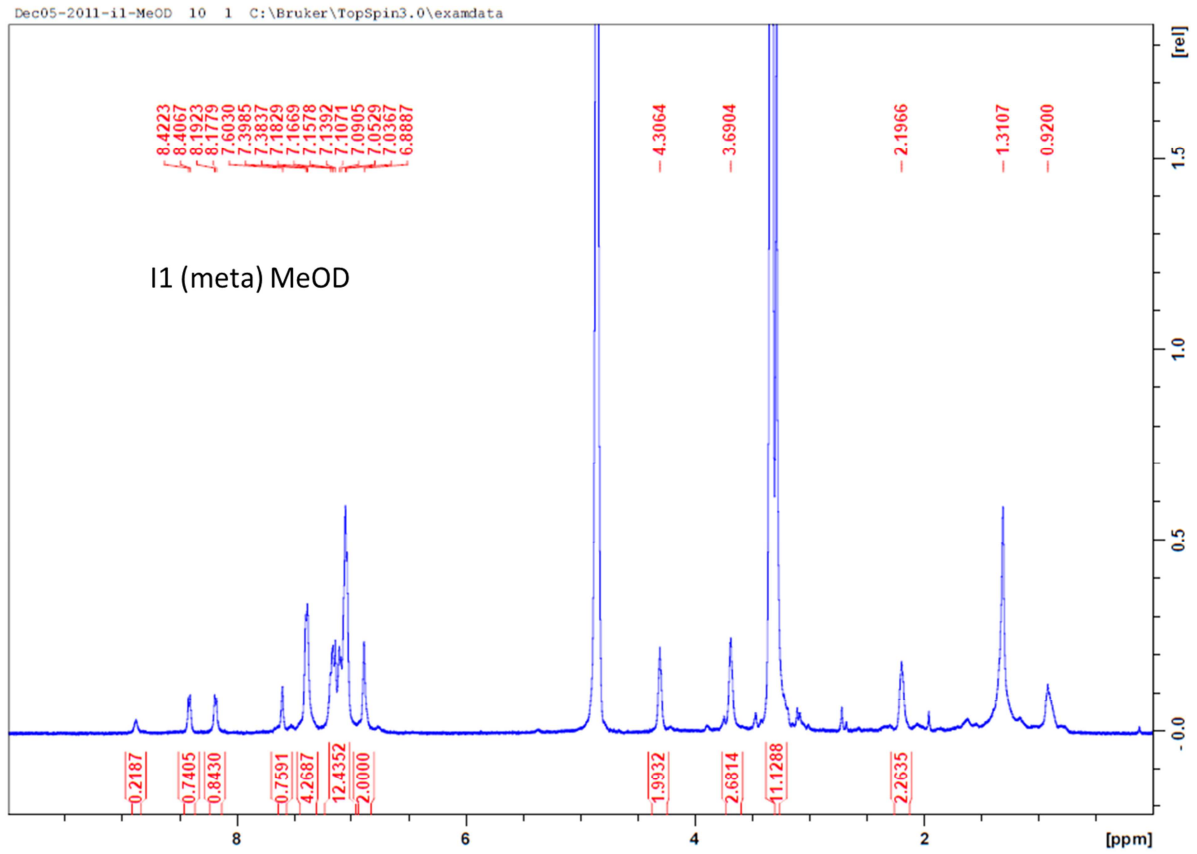
^{1}H NMR

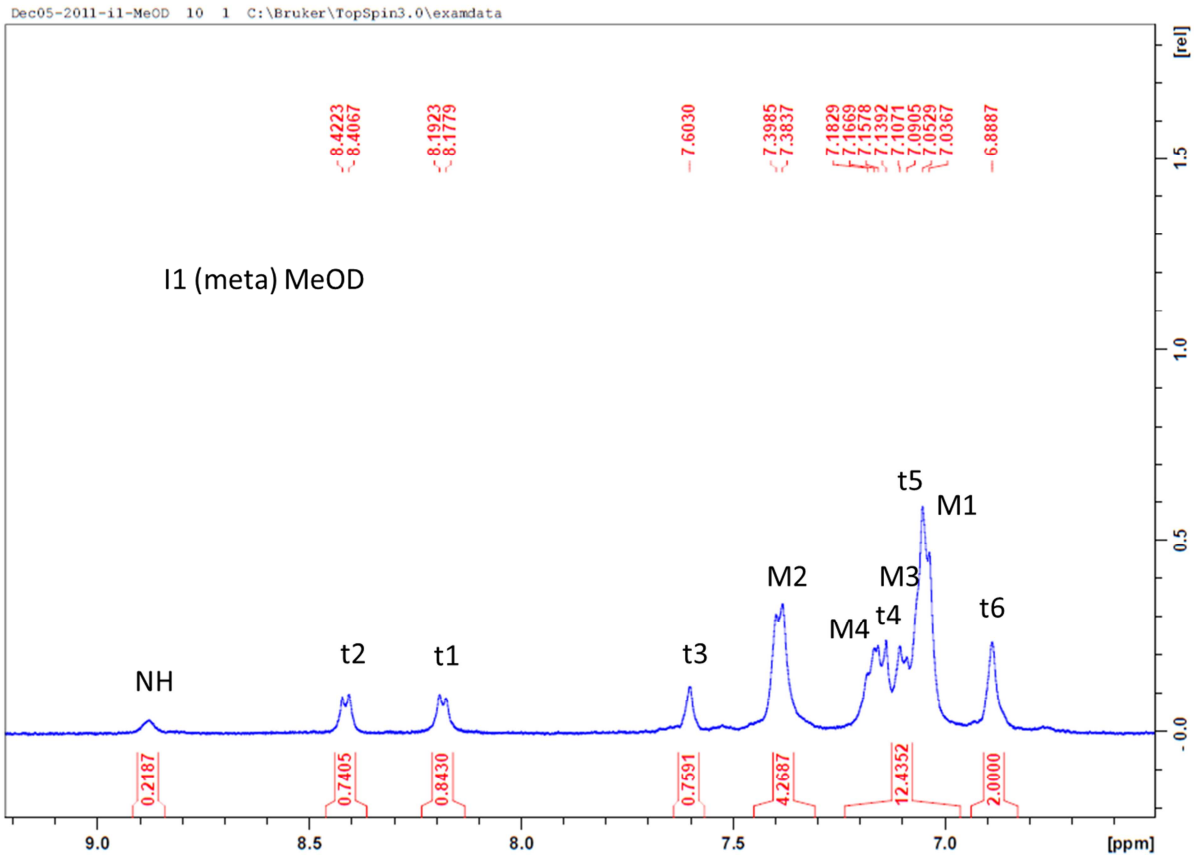


Signals assignment was done based on COSY and NOESY experiments (see below)

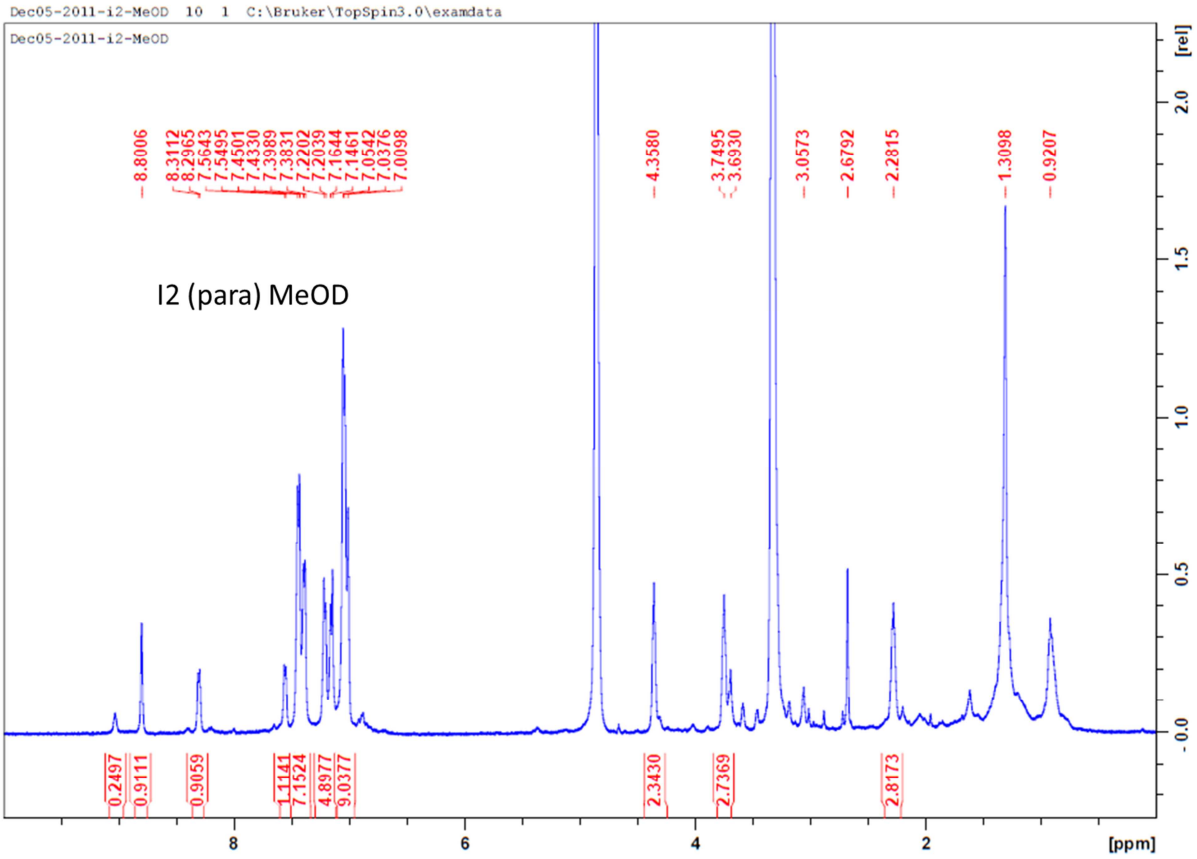


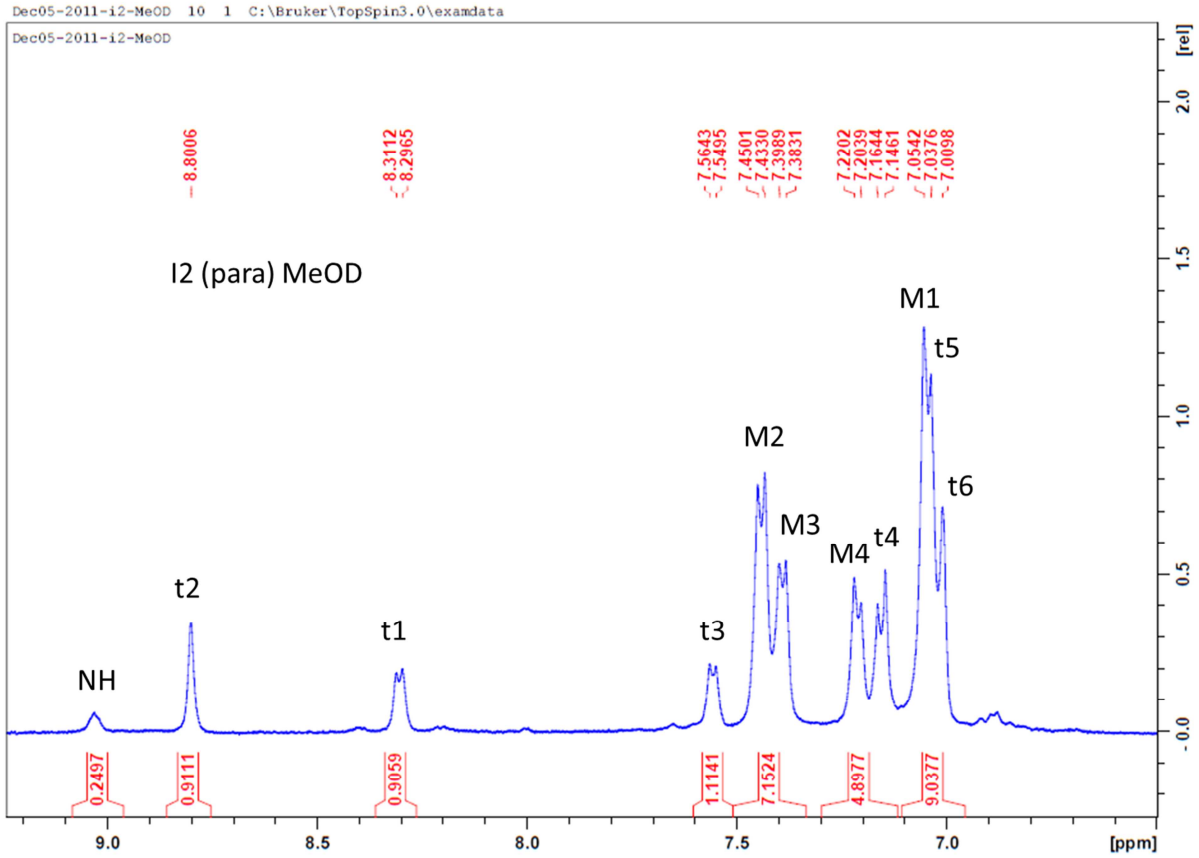
Signals assignment was done based on COSY and NOSY experiments (see below)


Dec05-2011-i1-MeOD 10 1 C:\Bruker\TopSpin3.0\examdata
8.4223
8.4067
8.1923
8.1779
7.6030
7.3985
7.3837
7.1829
7.1669
7.1578
7.1392
7.1071
7.0905
7.0529
7.0367
6.8887
4.3064
3.6904
2.1966
1.3107
0.9200
I1 (meta) MeOD
0.2187
0.7405
0.8430
0.7591
4.2687
12.4352
2.0000
1.9932
2.6814
11.1288
2.2635
[rel]
1.5
1.0
0.5
-0.0
8
6
4
2
[ppm]



Signals assignment was done based on COSY and NOESY experiments (see below)

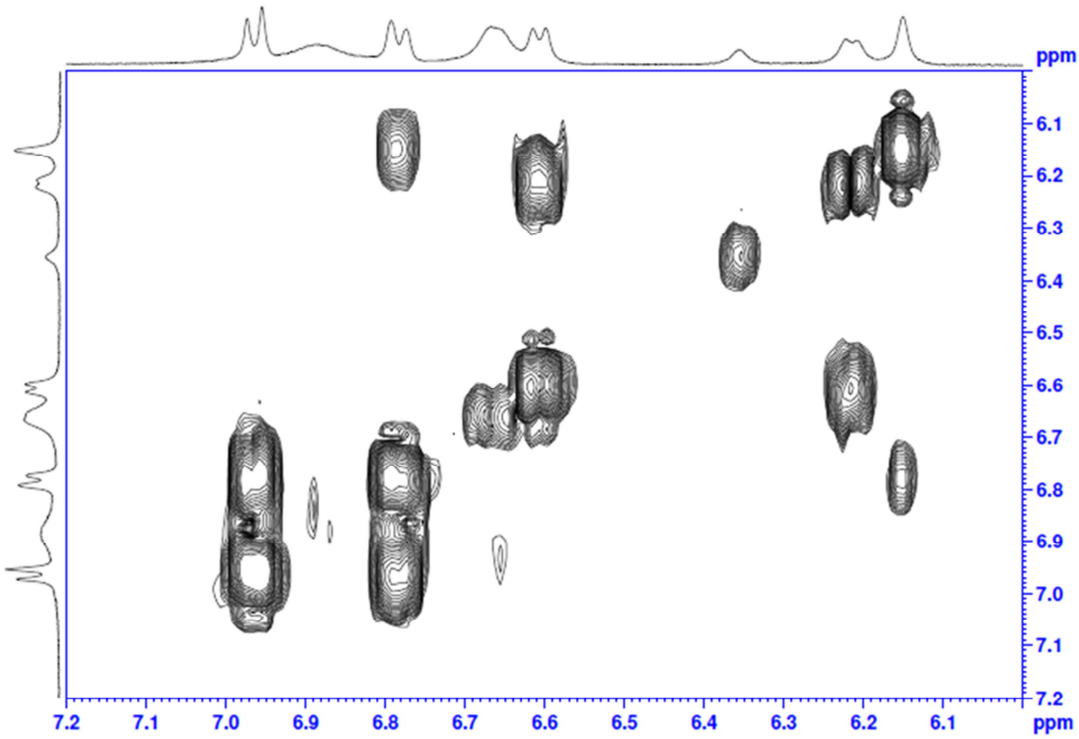

Dec05-2011-i2-MeOD 10 1 C:\Bruker\TopSpin3.0\examdata
Dec05-2011-i2-MeOD
I2 (para) MeOD
8.8006
8.3112
8.2965
7.5643
7.5495
7.4501
7.4330
7.3989
7.3831
7.2202
7.2039
7.1644
7.1461
7.0542
7.0376
7.0098
4.3580
3.7495
3.6930
3.0573
2.6792
2.2815
1.3098
0.9207
0.2497
0.9111
0.9059
1.1141
7.1524
4.8977
9.0377
2.3430
2.7369
2.8173
[rel]
[ppm]

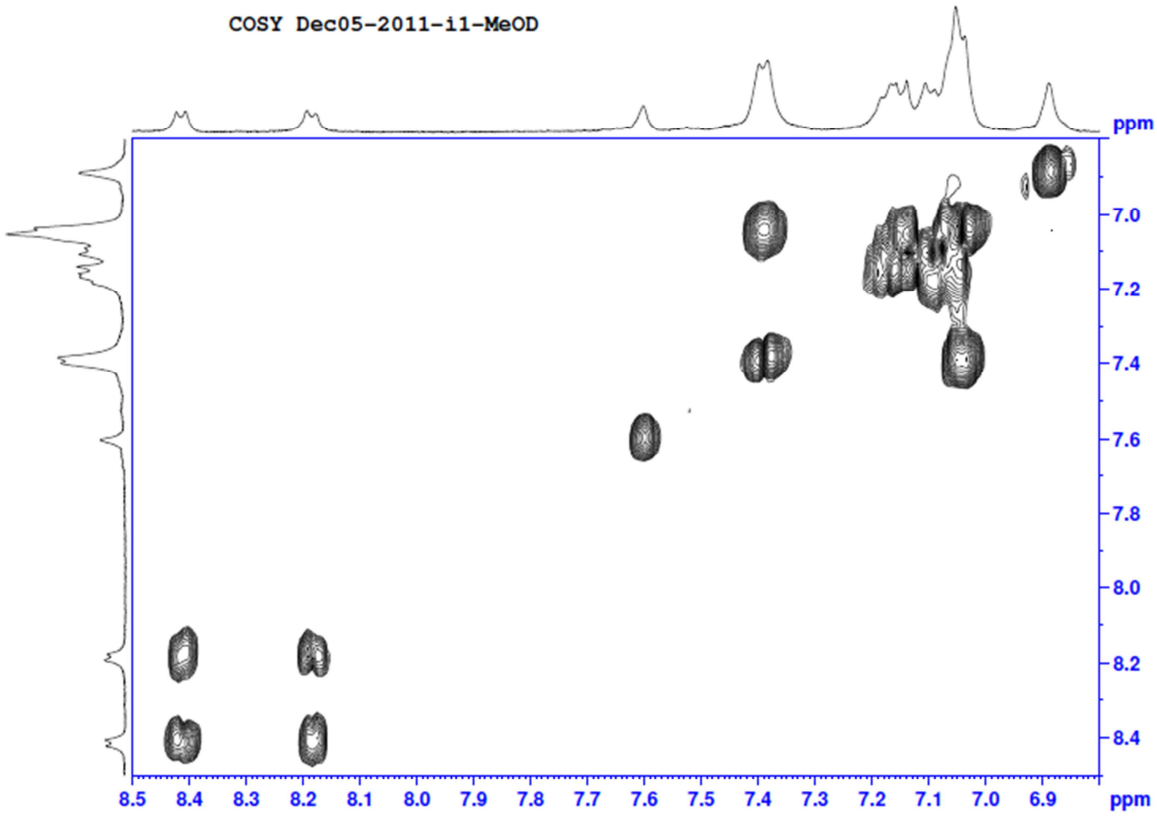


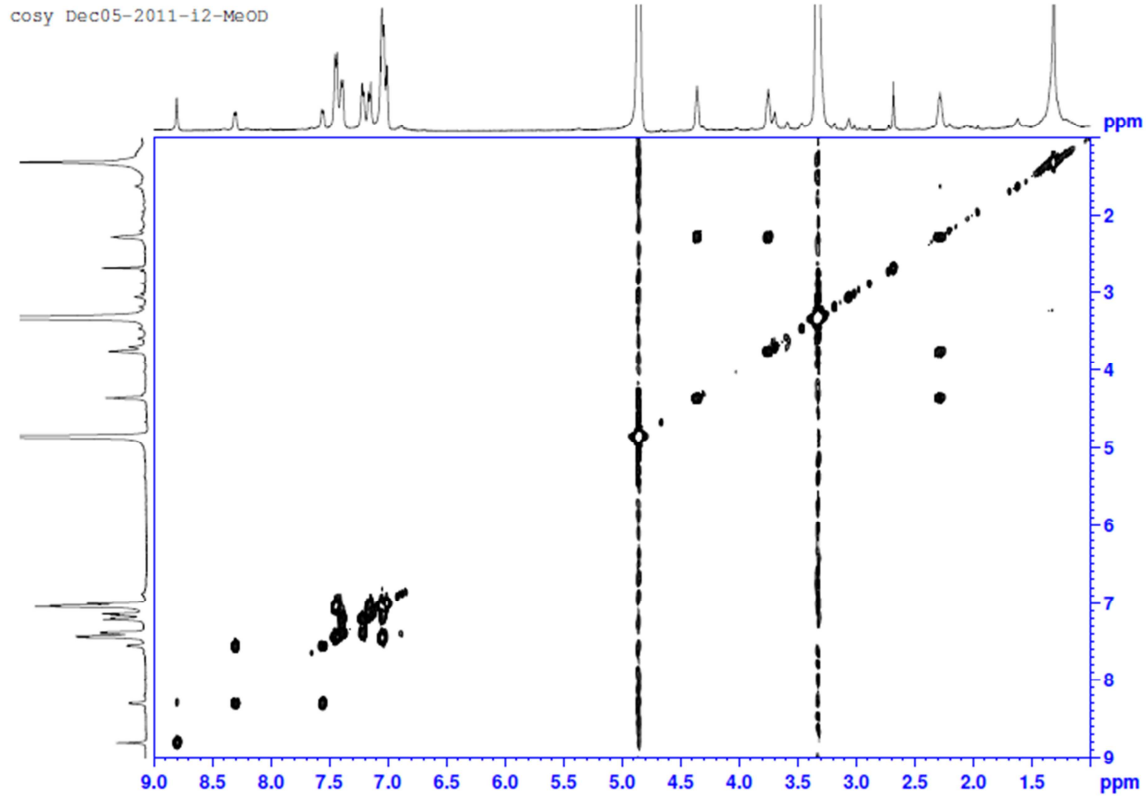
Signals assignment was done based on COSY and NOESY experiments (see below)

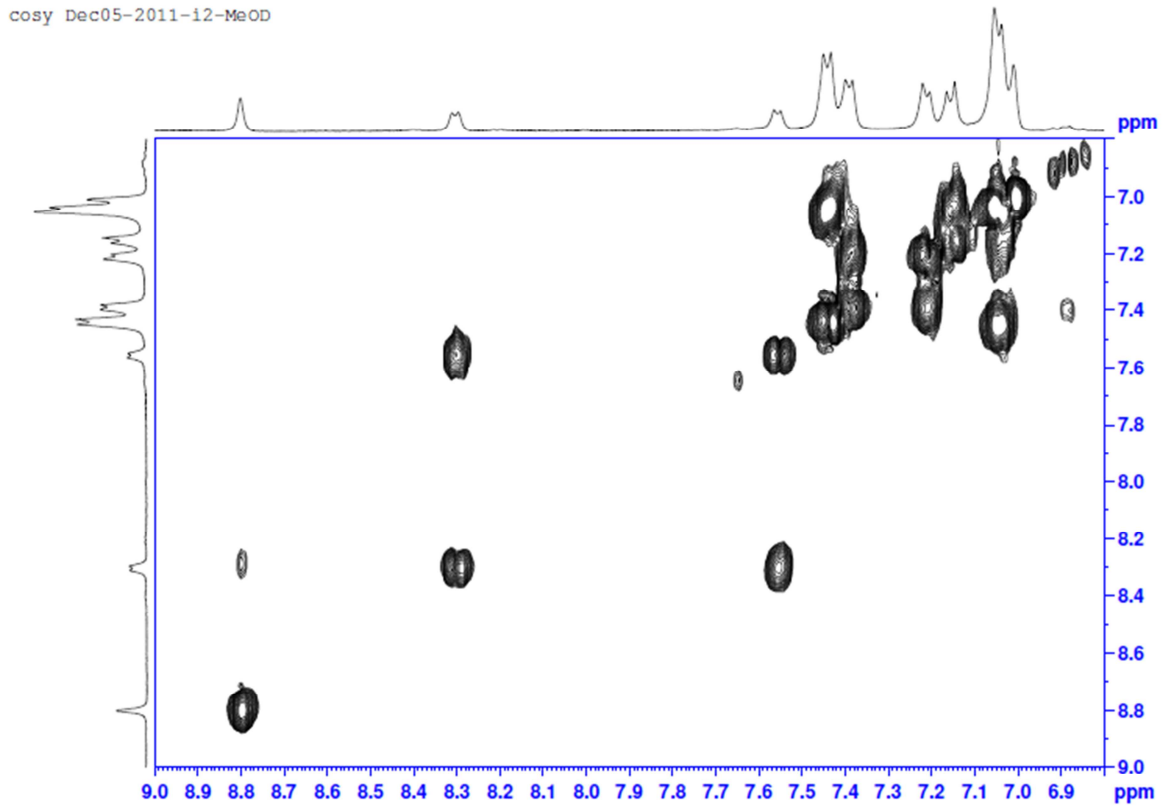
COSY

i1 D_2O COSY



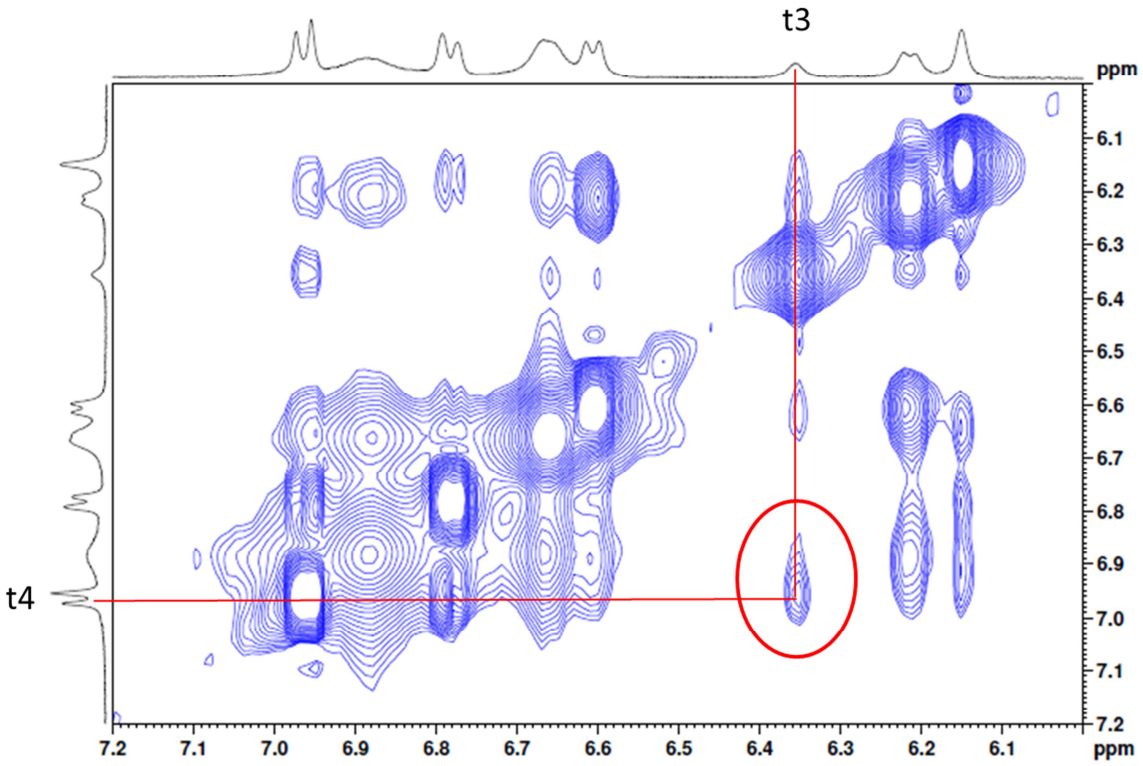

COSY Dec05-2011-i1-MeOD
ppm
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 6.9
ppm


cosy Dec05-2011-i2-MeOD
ppm
2
3
4
5
6
7
8
9
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5
ppm


cosy Dec05-2011-i2-MeOD
ppm
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
8.6
8.8
9.0
9.0 8.9 8.8 8.7 8.6 8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 6.9
ppm

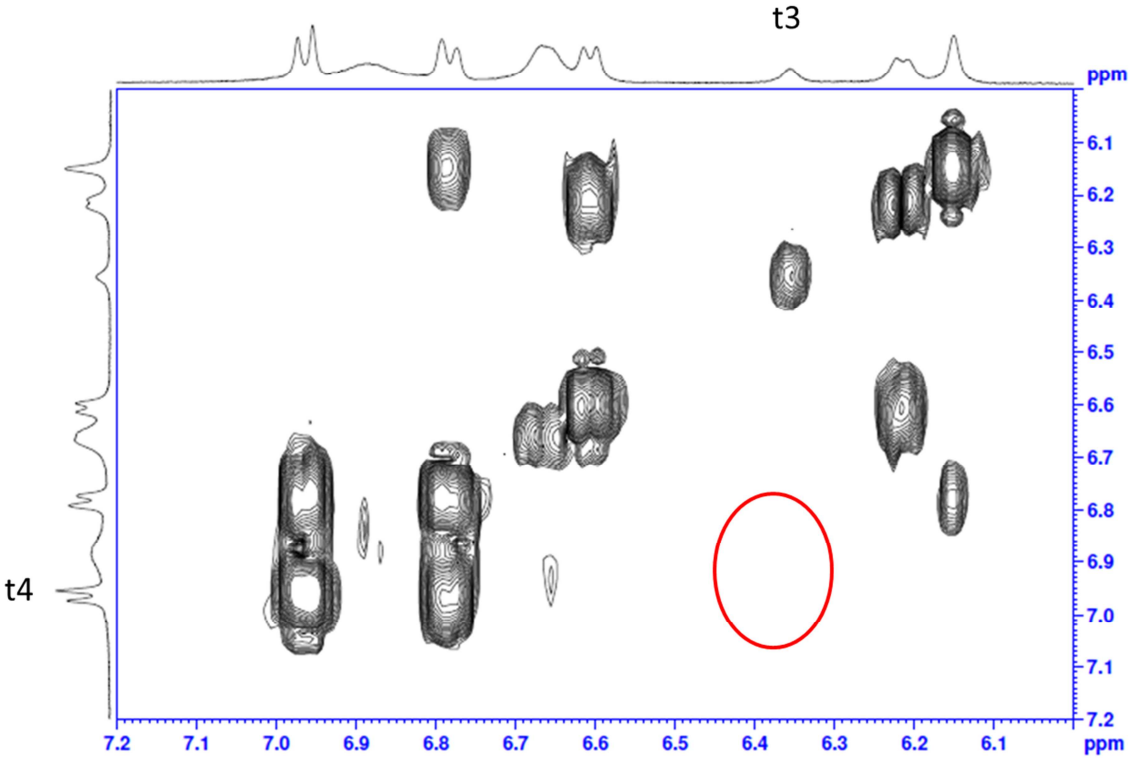
NOESY: i1 is a *meta* isomer

i1 is a meta isomer



NOESY i1 in D_2O

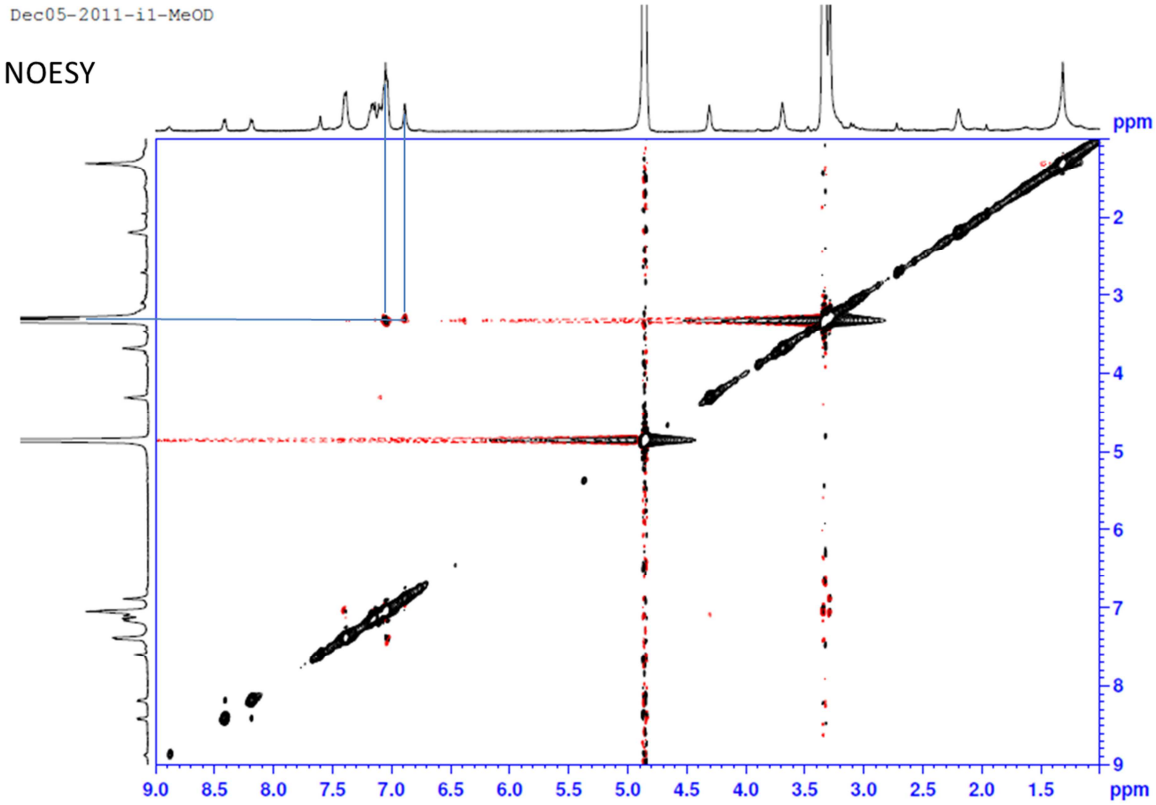
i1 is a meta isomer



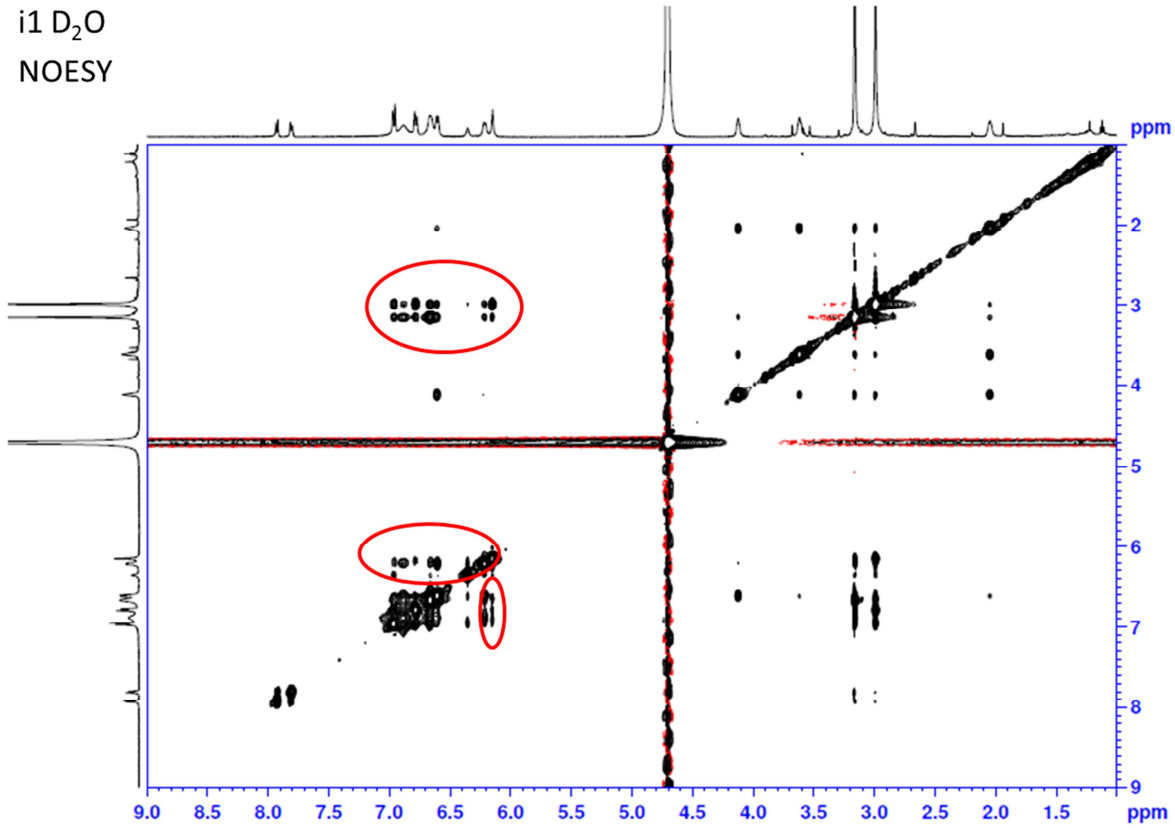
COSY i1 in D_2O: there is no t3 – t4 coupling

NOESY: intramolecular heterochromophore interaction in TMR-*meta*-MG dye in D_2O

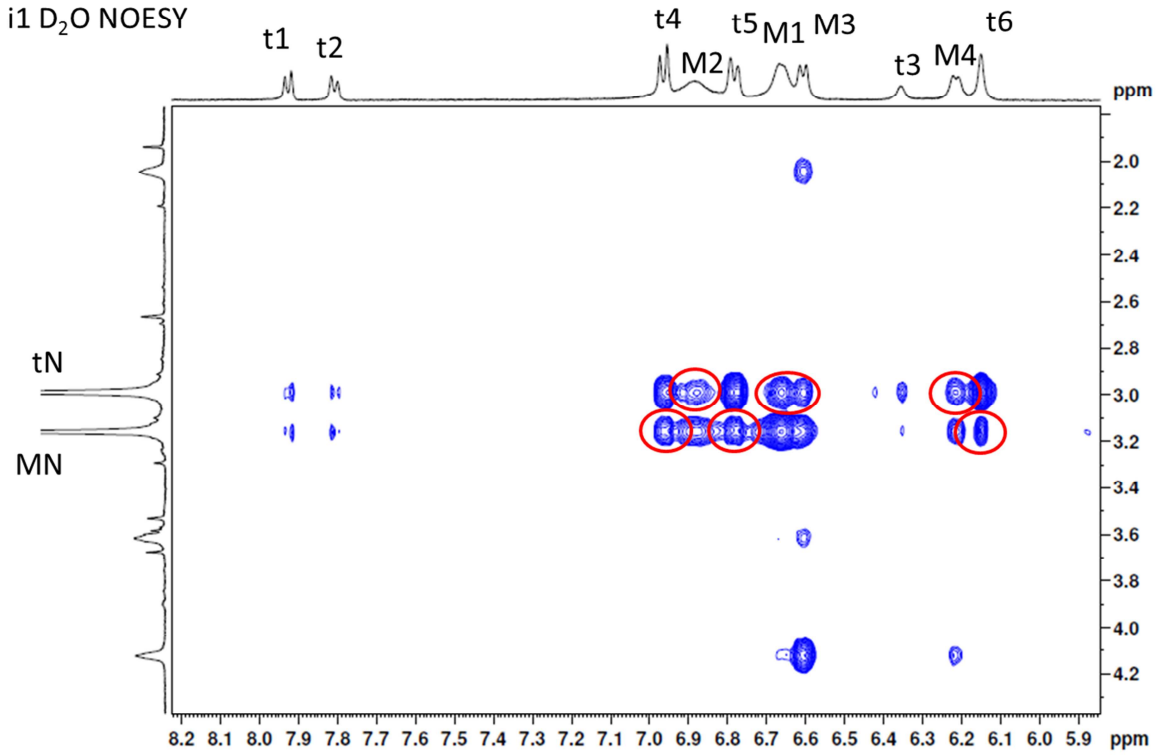
There is no much interaction between chromophores of i1 in MeOD



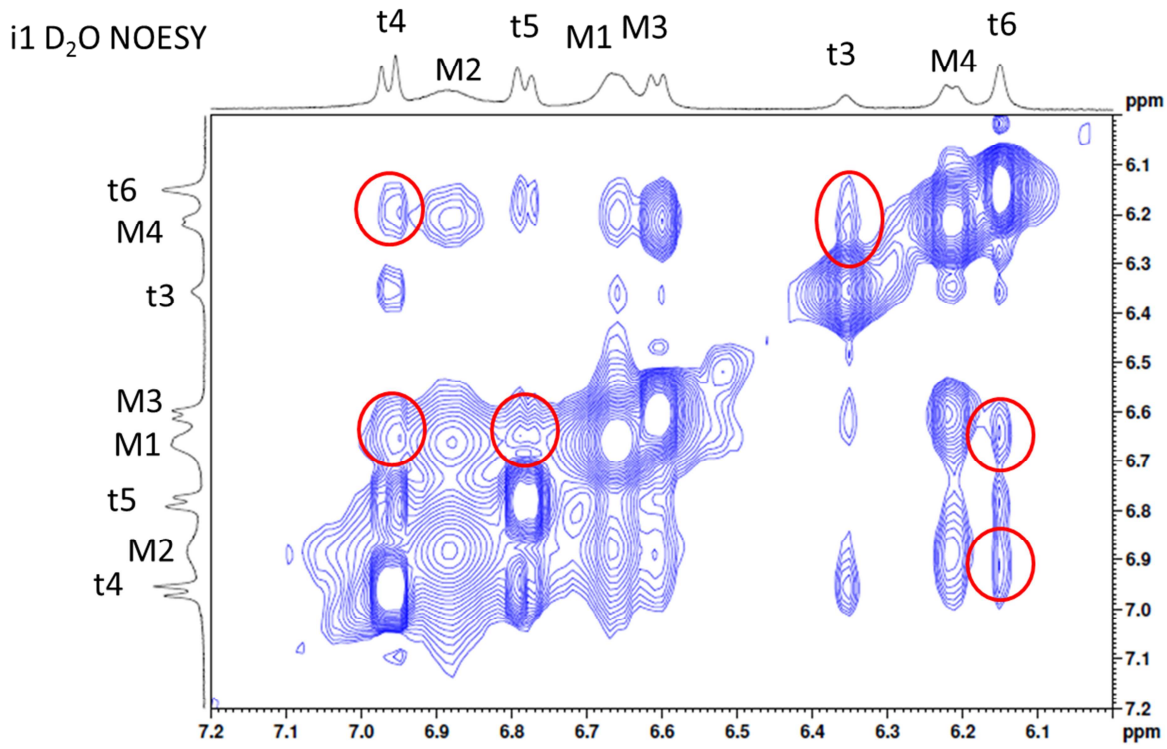
There is a pronounced interaction between two chromophores of i1 in D_2O



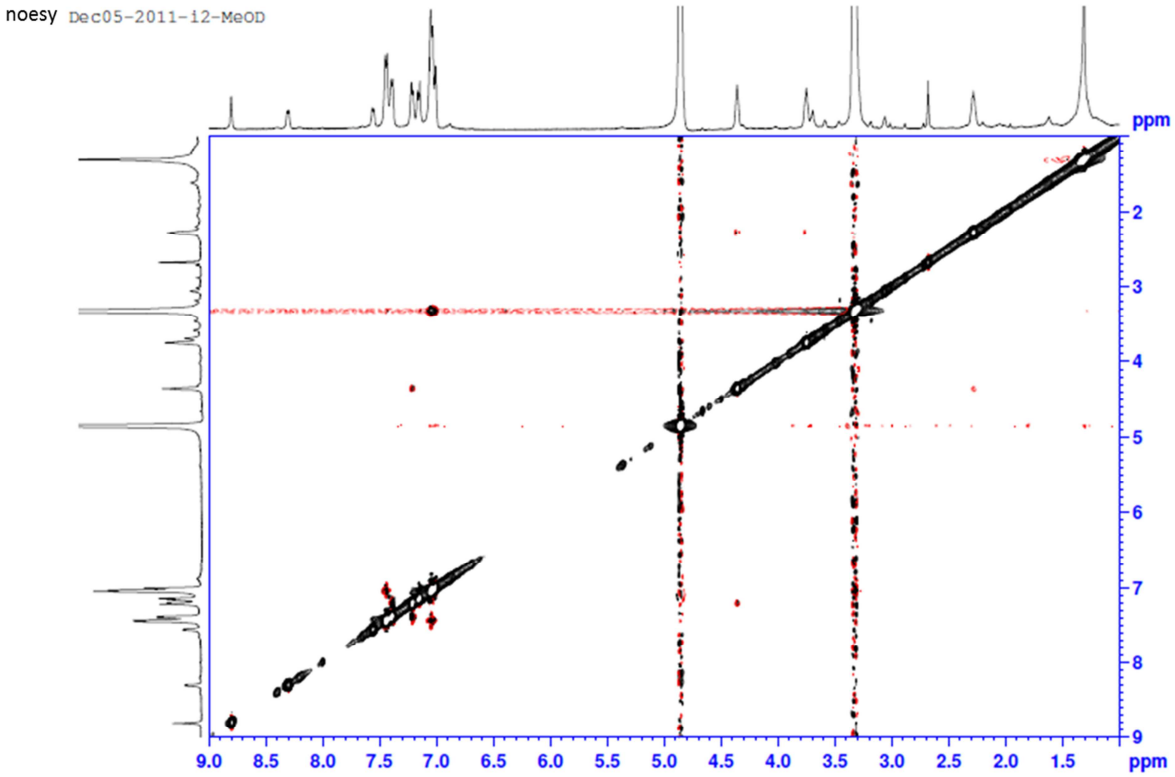
Methyl groups of TMR correlate with (are spatially close to) aromatics of MG and methyl groups of MG correlate with (are spatially close to) aromatics of TMR



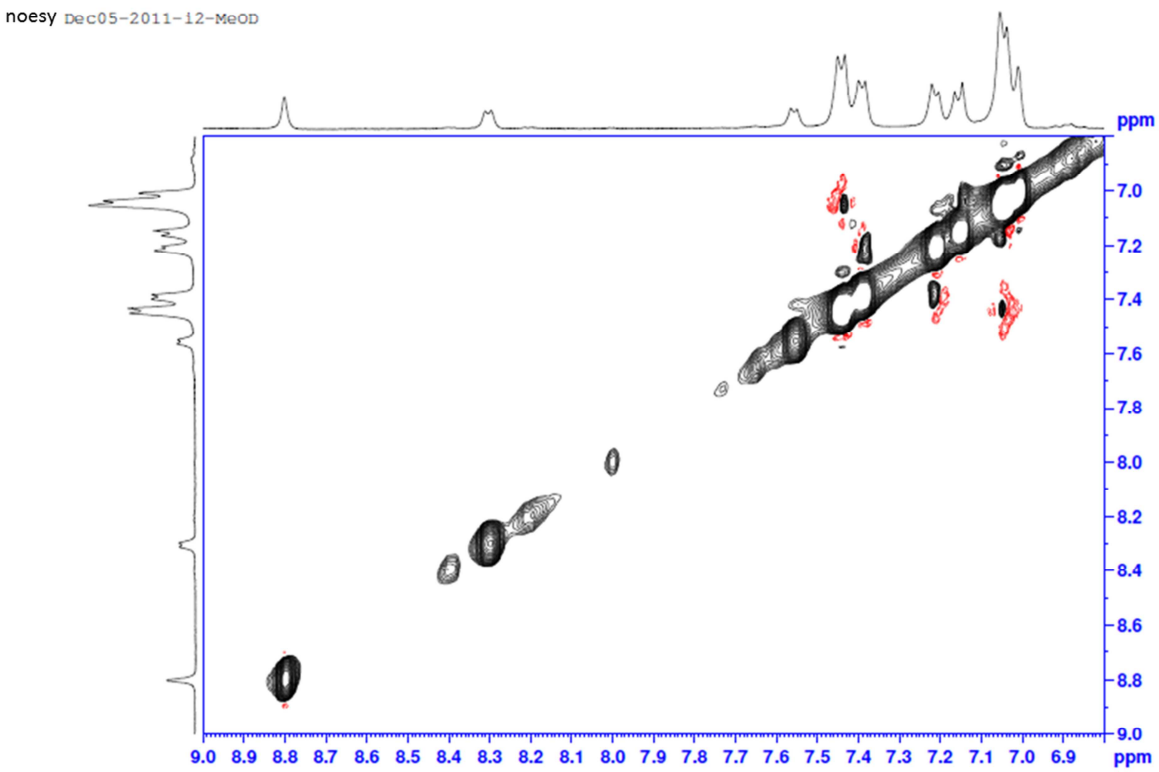
Aromatic protons of TMR correlate well with (are spatially close to) aromatic protons of MG



There is no much interaction between chromophores of i2 in MeOD



There is no much interaction between chromophores of i2 in MeOD



i2 (*para* isomer) is not soluble enough in D_2O to record its NMR. The higher solubility of *meta* isomer may serve as an additional proof of H-aggregate formation that involves interaction of aromatic hydrophobic moieties of both chromophores and as result leads to higher solubility of this molecule in hydrophilic D_2O compare to *para* isomer where this interaction is absent.

SI Movies

Movie 1. Z stacks of live-cell confocal microscopy images of the HEK 293 cells that express dL5 E52D L91S FAP fused to the transmembrane domain of B7-1 protein for inner-leaflet display after incubation with 150nM TMR-*para*-MG dye for 30min. Green channel corresponds to emission of TMR (575-630 nm) excited with 561 nm laser; red channel corresponds to emission of MG bound to FAP (650-710 nm) excited with 633 nm laser

Movie 2. Time series of live-cell spinning-disc images of the HEK 293 cells that express dL5 E52D L91S fused to the transmembrane domain of B7-1 protein for inner-leaflet display in the presence of 150nM TMR-*para*-MG after treatment with 100uM CCCP (8s/frame)

References

[1] C. Szent-Gyorgyi, B. F. Schmidt, J. A. Fitzpatrick, M. P. Bruchez, *J Am Chem Soc* **2010**, *132*, 11103.
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[3] C. Szent-Gyorgyi, B. F. Schmidt, Y. Creeger, G. W. Fisher, K. L. Zakel, S. Adler, J. A. Fitzpatrick, C. A. Woolford, Q. Yan, K. V. Vasilev, P. B. Berget, M. P. Bruchez, J. W. Jarvik, A. Waggoner, *Nat Biotechnol* **2008**, *26*, 235.
[4] T. L. Foley, A. Yasgar, C. J. Garcia, A. Jadhav, A. Simeonov, M. D. Burkart, *Org Biomol Chem* **2010**, *8*, 4601.