
CarboTag: a modular approach for live and functional imaging of plant cell walls

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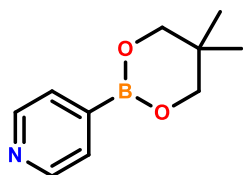
Table of contents

Synthetic procedures CarboTag, fluorophore precursors and CarboTag modified fluorophores	2-6
Spectroscopy	6
Figure S1, non-modified vs CarboTag modified fluorophore	7
Figure S2, plasmolysis	8
Figure S3, penetration of CW dyes (CT-AF488, CFW, SR2200)	9
Figure S4, penetration of propidium iodide	10
Figure S5, staining <i>E. coli</i>	11
Figure S6, staining <i>P. palmivora</i>	12
Microrheology	13-14
Figure S7, mesh size vs fluorescence lifetime	14
Figure S8, CT-BDP FLIM images ISX treatment	15
Figure S9, CT-BDP FLIM images FERONIA mutants	15
Figure S10, non-modified vs CarboTag modified Oregon Green	16
Figure S11, CT-OG FLIM images of the cell wall upon treatment	17
Figure S12, effect of pegylation on CT-Ox	18
Figure S13, timelapse mock treated roots with CT-Ox	19
Figure S14, staining of various plant structures	20
Figure S15, chemical structures non-functional CT-fluorophores	21
Figure S16, chemical structures functional CT-fluorophores	22
Figure S17, fluorescence spectra non-functional CT-fluorophores	23
Figure S18-S28, NMR spectra	24-34

Synthetic procedures CarboTag, fluorophore precursors and CarboTag modified fluorophores

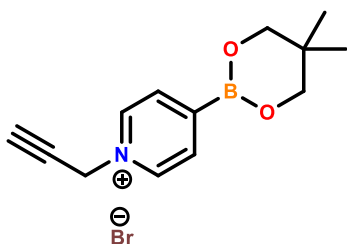
Vendors chemicals, dyes and treatments

All chemicals used for synthesis and treatments of biological material were purchased from Merck, TCI chemicals and AK Scientific. Fluorescent probes were purchased from Lumiprobe and Broadpharm. Silica (irregular, 40-63 μm particle size) and silica C18 (spherical, 20-45 μm , 100A) for flash and reverse phase chromatography respectively were purchased from Screening Devices, eluent mixture ratios are reported by volume. ^1H , ^{13}C and ^{19}F NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer.



Neopentyl glycol protected pyridine-4-boronic acid (1)

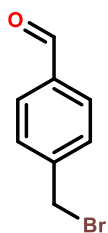
Pyridine-4-boronic acid (2.4 g, 20 mmol) and neopentyl glycol (2.2 g, 20 mmol) were dissolved in dry toluene (250 ml). The mixture was heated to 130 $^{\circ}\text{C}$ and refluxed overnight under N_2 . The mixture was cooled to r.t. The formed precipitate was collected by vacuum filtration, washed with toluene and dried under vacuum to yield 2.44 g of neopentyl glycol protected pyridine-4-boronic acid. Yield: 64 %. ^1H NMR (400 MHz, CDCl_3) δ 8.73 – 8.42 (m, 2H), 7.79 – 7.44 (m, 2H), 3.74 (s, 4H), 1.02 (s, 6H). ^{13}C NMR (101 MHz, CDCl_3) δ 149.14, 128.20, 72.55, 32.05, 22.00.



CarboTag (2)

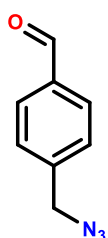
Neopentyl glycol protected pyridine-4-boronic acid (764 mg, 4 mmol) was added to 100 ml acetonitrile. The mixture was purged with N_2 for 5 minutes before the dropwise addition of propargyl bromide (80% in toluene, 8.6 ml, 80 mmol). The mixture was left stirring for 48 h at r.t. under N_2 . The precipitate was removed by filtration and filtrate was evaporated to dryness. The resulting brown solid was dried overnight to yield 989 mg of CarboTag. Yield: 80%. ^1H NMR (400 MHz, D_2O) δ 8.89 (d, J = 6.2 Hz, 2H), 8.26 (d, J = 6.2 Hz, 2H), 5.50 (d, J = 2.6 Hz, 2H), 3.39 (s, 5H), 3.28 (s, 1H), 0.86 (s, 7H). ^{13}C NMR (101 MHz, D_2O) δ 141.08, 130.60, 118.18, 79.13, 72.85, 67.27, 49.10, 35.15, 19.49.

4(bromomethyl)-benzaldehyde (3)



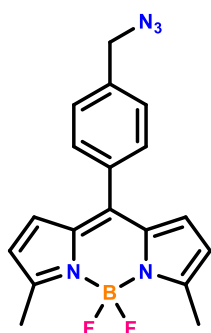
4(bromomethyl)benzonitrile (10 g , 51 mmol) was added to 100 ml of dry toluene and cooled to 0 °C in an ice bath. Add 72 ml DIBAL-H in hexane (1M, 72 mmol) over a period of 2h (6x12 ml) at 0 °C. The solution was left stirring for 1h at 0 °C before being diluted with 150 ml chloroform. 330 ml 10% HCl was slowly added, the mixture was allowed to heat up to r.t. and was left stirring overnight. The organic layer was isolated and washed with 3x 125 ml water, dried over MgSO₄ and concentrated to yield a white solid. The solid was recrystallized from hexane to yield 6.79 g of white, fine needle like crystalline solid. Yield: 67%. **¹H NMR** (400 MHz, CDCl₃) δ 10.02 (s, 1H), 7.87 (d, J = 8.1 Hz, 2H), 7.56 (d, J = 7.7 Hz, 3H), 4.52 (s, 2H). **¹³C NMR** (101 MHz, CDCl₃) δ 191.64, 144.39, 136.29, 130.07 (d, J = 49.5 Hz), 32.09.

4(azidomethyl)-benzaldehyde (4)



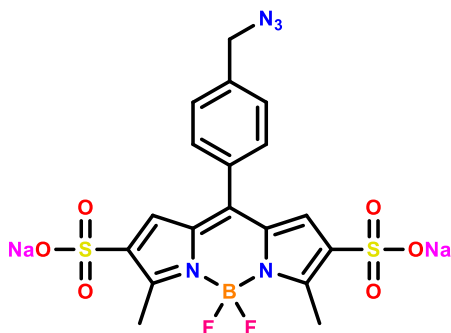
4-(bromomethyl)benzaldehyde (5g, 25 mmol) and NaN₃ (2.5 g, 38 mmol) were added to 50 mL of DMF in a round bottom flask. The mixture was stirred for 1.5 hours at 60 °C. After cooling the solution was diluted with 250 mL of ethyl acetate and washed with 2x250mL of water. The organic layer was dried with MgSO₄, filtered, concentrated and dried under vacuum to yield 4-(azidomethyl)benzaldehyde as an oil with pleasant smell (3.76 g, 93% yield). **¹H NMR** (400 MHz, CDCl₃) δ 10.02 (s, 1H), 7.90 (d, J = 8.1 Hz, 2H), 7.49 (d, J = 8.5 Hz, 2H), 4.45 (s, 2H). **¹³C NMR** (101 MHz, CDCl₃) δ 191.74, 142.24, 136.30, 130.32, 128.59, 54.38.

N₃-BODIPY porosity probe (5)



4-(azidomethyl)benzaldehyde (2.0 g, 12.41 mmol) was added to a 1L 3-neck round bottom flask with 500 mL of anhydrous dichloromethane and 2-methylpyrrole (2.092 mL, 24.82 mmol). The solution was purged with N₂ for 30 minutes after which trifluoroacetic acid (500 μ L, 6.2 mmol) was added dropwise. The mixture turned orange and was stirred for 2 hours after which 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (2.82 g, 12.41 mmol) was added followed by purging with N₂ for 10 minutes followed by 20 minutes of stirring. Di-isopropylethylamine (15.1 mL, 86.7 mmol) and boron trifluoride diethyl etherate (15.3 mL, 124 mmol) were added, the mixture was left stirring for 24 hours under N₂. The reaction mixture was diluted with 2:3 hexane:ethyl acetate followed by removal of dichloromethane under reduced pressure. After purification on silica (2:3 hexane:ethyl acetate) the product was isolated as a red, crystalline solid (1.07 g, 25% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.52 (d, J = 8.1 Hz, 2H), 7.43 (d, J = 8.1 Hz, 2H), 6.69 (d, J = 4.1 Hz, 2H), 6.27 (d, J = 4.1 Hz, 2H), 4.46 (s, 2H), 2.65 (s, 6H). ¹⁹F NMR (376 MHz, CDCl₃) δ -147.61 (dd, J = 65.3, 32.1 Hz).

Sulfonated N₃-BODIPY porosity probe (6)



N₃-BODIPY (180 mg, 0.52 mmol) was dissolved in 24 mL of dry DMF after which sulfur trioxide pyridine complex is added (1.632 g, 10 mmol). The mixture was purged with N₂ for 15 minutes and heated to 60 °C for 24 hours after which it was cooled down to r.t. A saturated sodium carbonate solution was added until no more gas formation was observed. The resulting aqueous solution is washed with 2x 25 mL chloroform and concentrated. To remove the final traces of pyridine 40 mL of toluene was added and evaporated under reduced pressure, this was repeated 3 times. The resulting solid was dissolved in absolute ethanol, filtered through a celite pad and the filtrate concentrated and dried under vacuum to yield 245 mg of sulfonated N₃-BODIPY with acceptable purity. Yield: 85%. ¹H NMR (400 MHz, D₂O) δ 7.59 (d, J = 5.3 Hz, 4H), 7.20 (s, 2H), 4.55 (s, 2H), 2.65 (d, J = 1.2 Hz, 6H).

General synthesis protocol CarboTag modified fluorophores

Prepare a 0.5 M CarboTag solution in MilliQ (MQ) water and a 50 mM tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) solution in DMF. Add copper(I) bromide (CuBr) (6 mg, 0.04 mmol) to a vial equipped with a stirring bar. Add 1 ml of TBTA solution (0.05 mmol) and stir for 5 min. Add additional DMF to ensure the final DMF:H₂O ratio is 2:1 if needed. Add a concentrated solution of the fluorophore (H₂O or DMF) and add 2 equivalents of CarboTag for every equivalent of dye. Add additional water to have a DMF:H₂O ratio of 2:1. Seal with a septum and bubble with N₂ for 15 min, stir overnight at r.t. Dilute the solution with MQ water, filter out the precipitate with a 0.45 µm syringe filter. Freeze dry the filtered solution, redissolve in water and further dilute based on absorbance.

Comment on general synthesis protocol

Preparing separate solution of CarboTag, TBTA and the fluorophore allows for a simpler procedure since weighing milligrams these compounds dry is tedious and prone to error. Additionally, dissolving these compounds separately prevents precipitation and/or slow dissolving opposed to adding solvent to a mixture of dry reagents. Since copper(I) bromide and TBTA are both insoluble in water adding all DMF containing solutions before adding H₂O prevents these compounds from precipitating. Their poor solubility is later exploited for purification by diluting the reaction mixture with water and removing the precipitate consisting of CuBr and TBTA while the desired product remains in solution. The quantity of CuBr and TBTA is kept constant independent of the amount of dye and reaction volume, at our largest scale synthesis (CarboTag-AF430) 25 mg of dye was used which amounts to a total of 0.032 mmol. This excess of catalyst and ligand makes changing their respective used quantities redundant. In our protocol we observed occasional precipitation of TBTA upon the addition of water, to prevent this a DMF:water ratio of 3:1 instead of 2:1 can be used. This should impact the overall efficiency of the synthesis minimally.

CarboTag-AF430

The general synthesis protocol was followed, AF430-azide (25 mg, 0.032 mmol) was dissolved in 0.8 ml MQ water. 0.128 ml 0.5M (0.064 mmol) CarboTag solution was added. The total reaction volume was 3 ml.

CarboTag-AF488

The general synthesis protocol was followed, AF488-azide (20 mg, 0.029 mmol) was dissolved in 2 ml MQ water. 0.12 ml 0.5M (0.060 mmol) CarboTag solution was added. The total reaction volume was 6 ml.

CarboTag-sCy3

The general synthesis protocol was followed, sCy3-azide (10 mg, 0.0136 mmol) was dissolved in 1 ml MQ water. 0.054 ml 0.5M (0.0272 mmol) CarboTag solution was added. The total reaction volume was 3 ml.

CarboTag-sCy5

The general synthesis protocol was followed, sCy5-azide (10 mg, 0.0132 mmol) was dissolved in 1 ml MQ water. 0.052 ml 0.5M (0.0264 mmol) CarboTag solution was added. The total reaction volume was 3 ml.

CarboTag-BDP

Sulfonated N3-BODIPY porosity probe (50 mg, 0.09 mmol) and CarboTag (27.9 mg, 0.09 mmol) were dissolved in 2 ml water-DMF (1:3) and bubbled with N₂ for 5 min. TBTA (9.5 mg, 0.018 mmol), CsF (27.3 mg, 0.18 mmol) and CuBr (5.2 mg, 0.036 mmol) were added and the mixture was left stirring for 5 h. The mixture was concentrated under vacuum and purified on a C18 reverse phase silica flash column using a 5:95 to 20:80 methanol-water gradient.

CarboTag-OG

The general synthesis protocol was followed, OG-azide (5 mg, 0.009 mmol) was dissolved in 1 ml DMF. 0.036 ml 0.5M (0.018 mmol) CarboTag solution was added. The total reaction volume was 3 ml.

CarboTag-Ox

Azido-PEG₈-amine (8.6 mg, 0.0195 mmol), di-isopropylethylamine (2.5 mg, 0.195 mmol) and BDP581/591-NHS ester were added to 1 ml DMF, degassed with N₂ and left stirring overnight. 6 mg CuBr was added followed by 1 ml 50 mM TBTA in DMF. 0.52 ml 0.5 M CarboTag in MQ water was added followed by 0.95 ml MQ water. The reaction was left stirring overnight. After diluting with MQ water the resulting solution was filtered through a filter paper instead of a syringe filter.

CarboTag-BDP581/591

The general synthesis protocol was followed, BDP581/591-azide (5 mg, 0.01 mmol) was dissolved in 0.4 ml DMF. 0.04 ml 0.5M (0.02 mmol) CarboTag solution was added. The total reaction volume was 3 ml.

Spectroscopy

Steady state fluorescence

UV-Vis absorption measurements were recorded on a Shimadzu UV2600 Spectrophotometer using 1 cm path quartz cuvettes. Fluorescence emission and excitation measurements were performed on a Agilent Cary Eclipse Fluorescence spectrophotometer with a xenon lamp using 10 mm quartz cuvettes. Absorption of samples for fluorescence emission and excitation measurements were kept below 0.1.

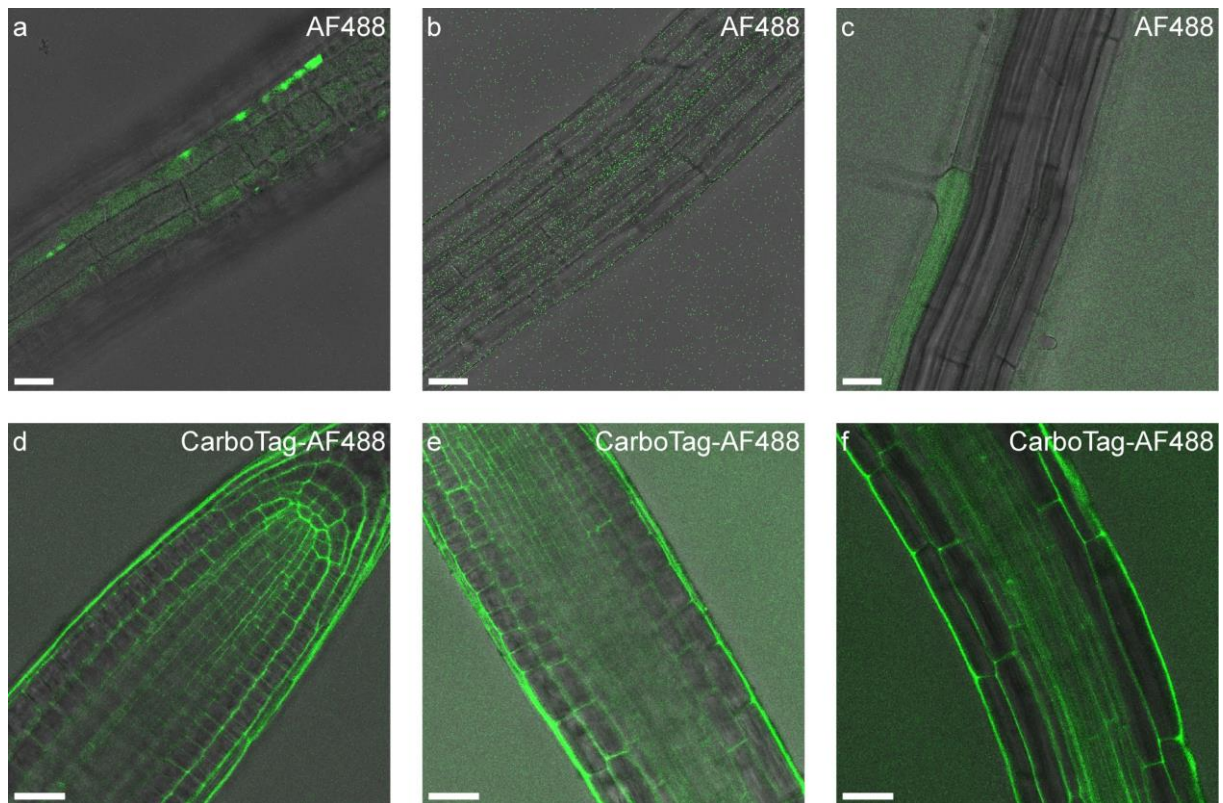


Figure S1: a-c) Arabidopsis roots stained with non-functionalized Alexafluor488 (AF488) show limited staining. d-f) CarboTag-AF488 exhibits superior staining over its non-modified counterpart. Scale bars represent 25 μm

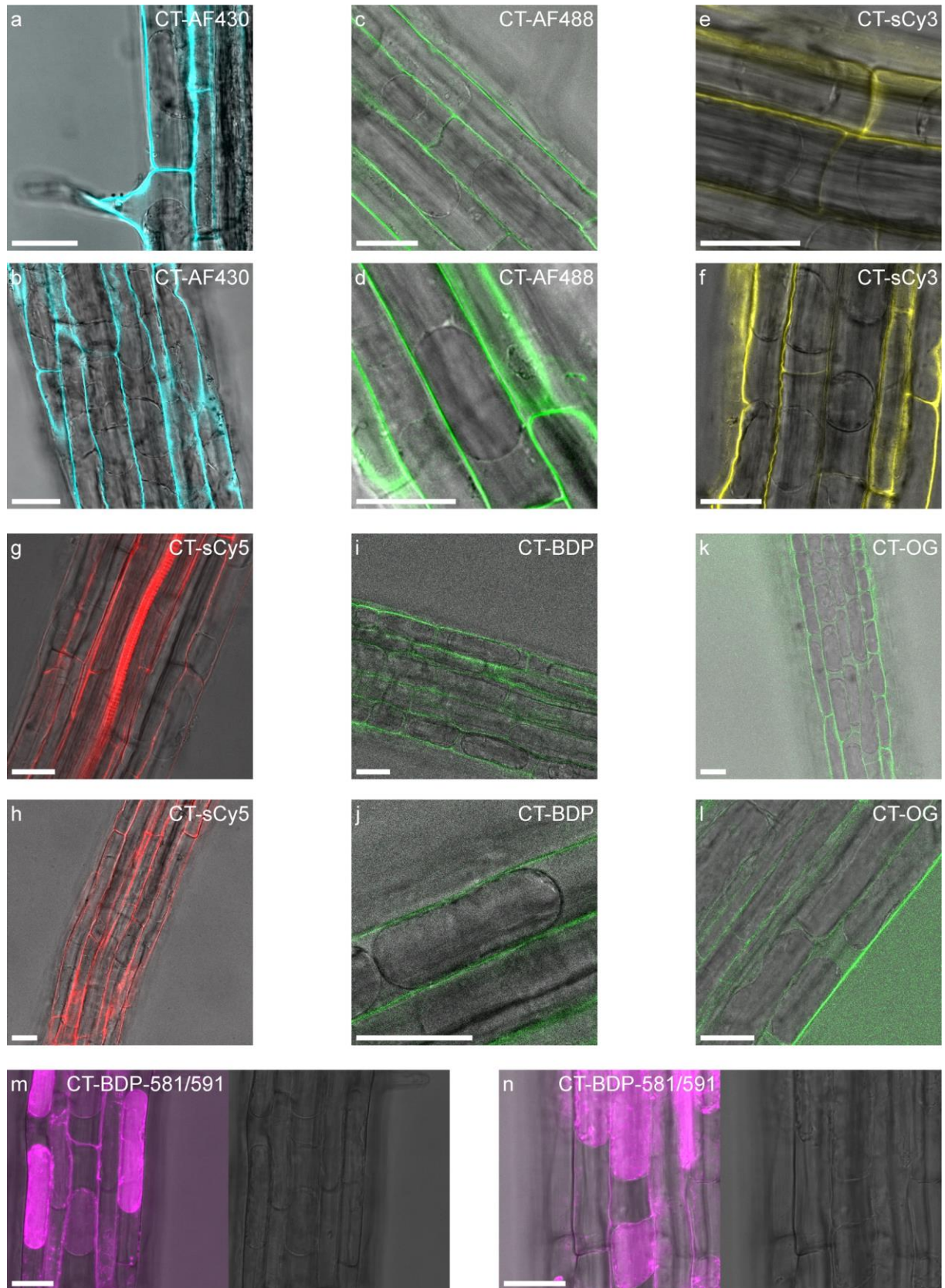


Figure S2: plasmolysis of roots incubated with 40 μ M CT-AF430 (a,b), CT-AF488 (c,d), CT-sCy3 (e,f), CT-sCy5 (g,h), CT-BDP (i,j), CT-OG (k,l) and 10 μ M CT-Ox (m,n) treated with 0.5 M mannitol. Scale bars represent 25 μ m

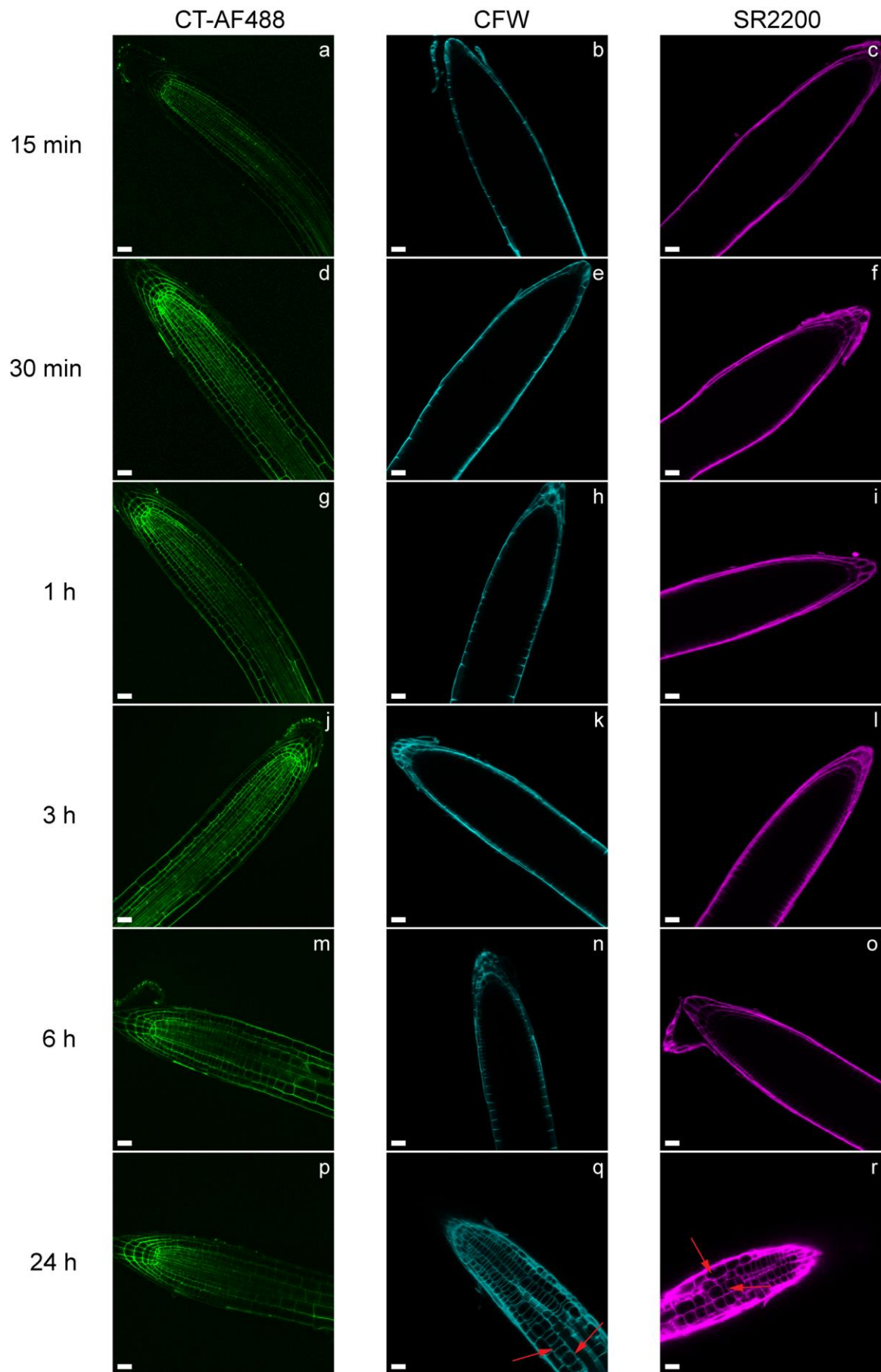


Figure S3: penetration of 40 μM CarboTag-AF488 (CT-AF488, green), 50 $\mu\text{g/ml}$ Calcofluor White (CFW, cyan) and 1x1000 diluted Renaissance 2200 (magenta, SR2200) over time. Red arrows (q,r) indicate altered cell shapes caused by 24 h exposure to CFW and SR2200. Scale bars represent 25 μm .

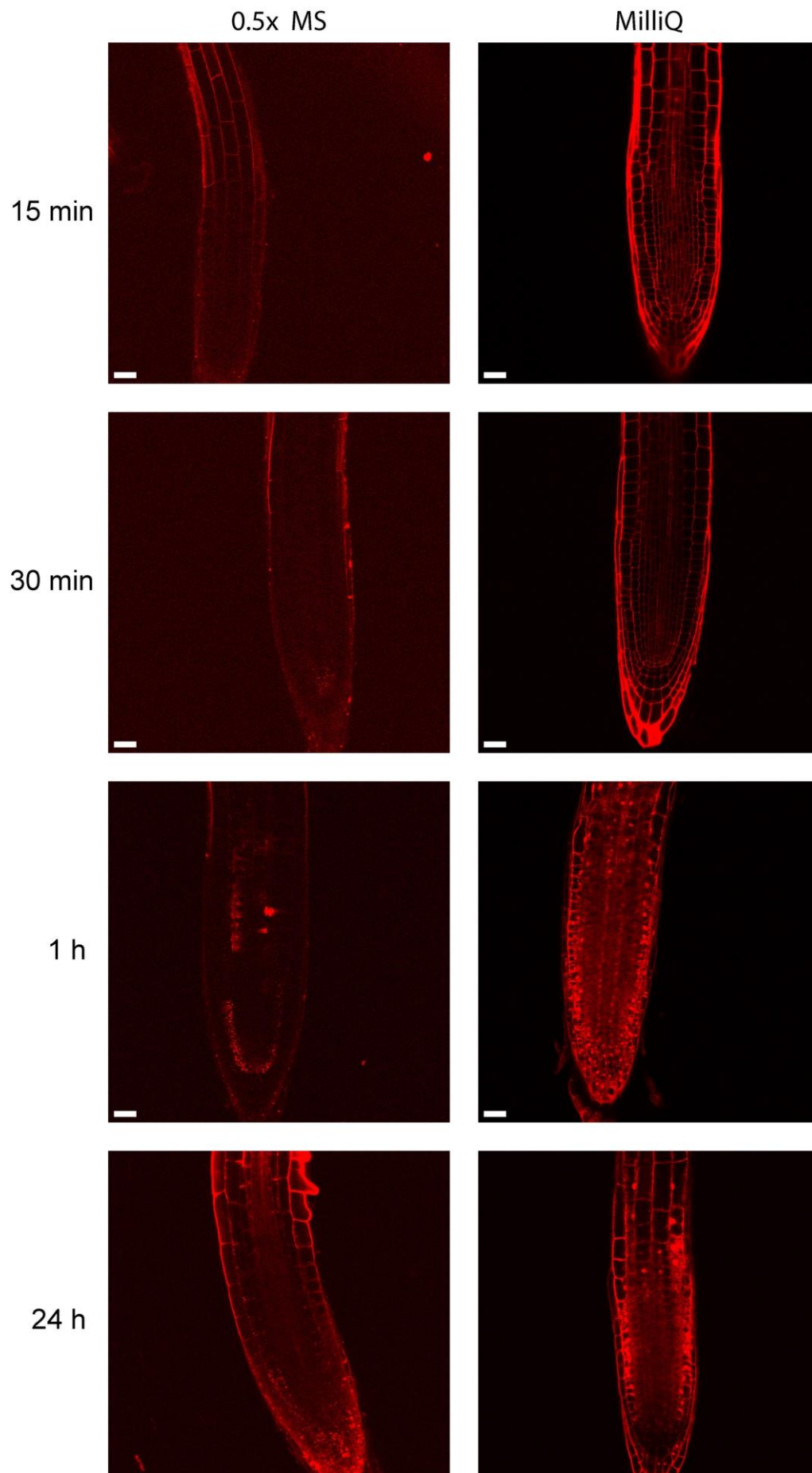


Figure S2: penetration of 15 μ M propidium iodide (PI, red) over time in 0.5x MS and MilliQ water. Scale bars represent 25 μ m.

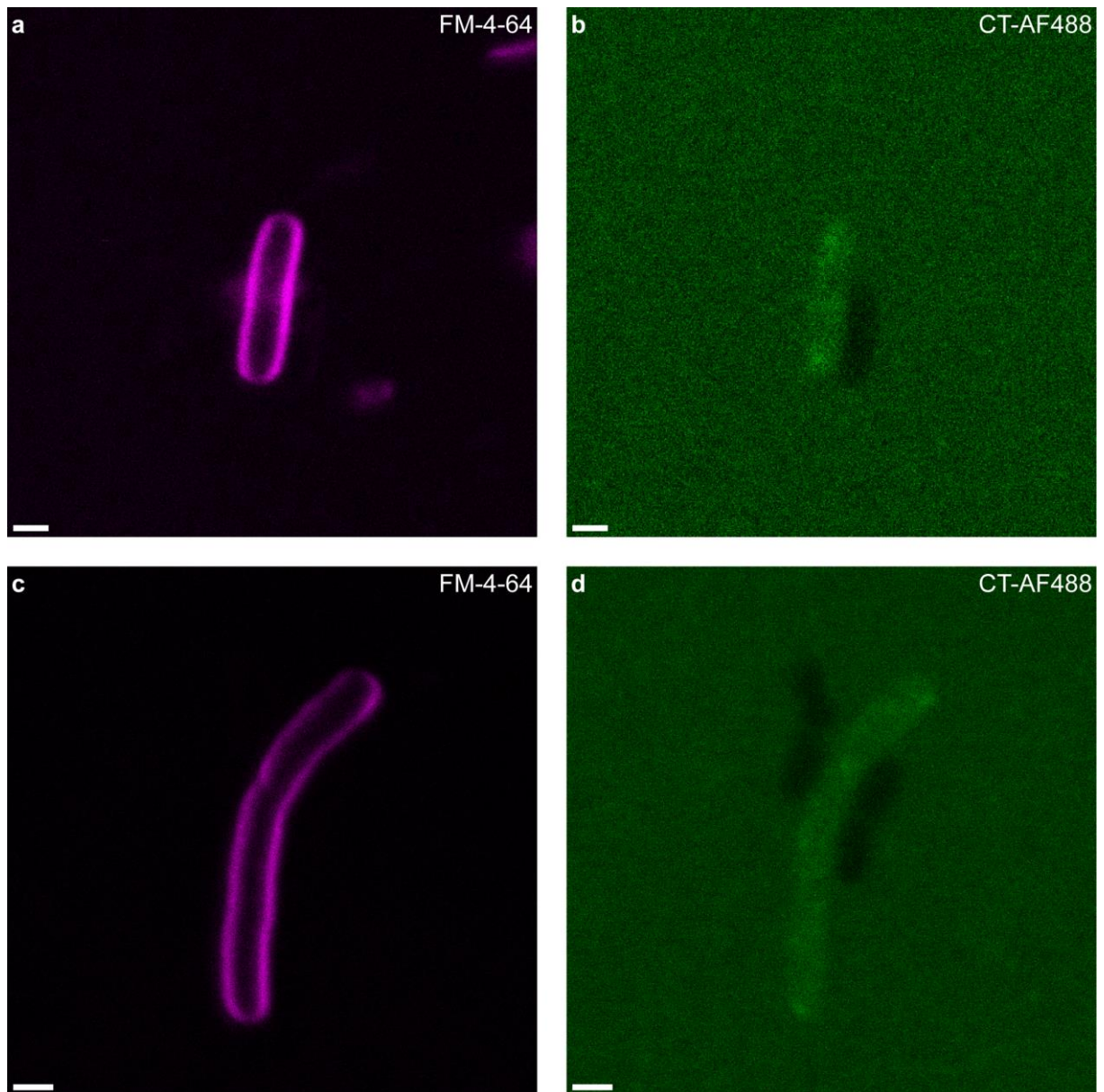


Figure S5: a-d) *E. coli* multicolor staining with FM-4-64 (0.5 mg/ml, magenta) and CT-AF488 (10 μM green). FM-4-64 signal from the membrane is visible (a,c) CT-AF488 does not produce a clear outline of the bacterial cell wall (b,d). Scale bars represent 1 μm.

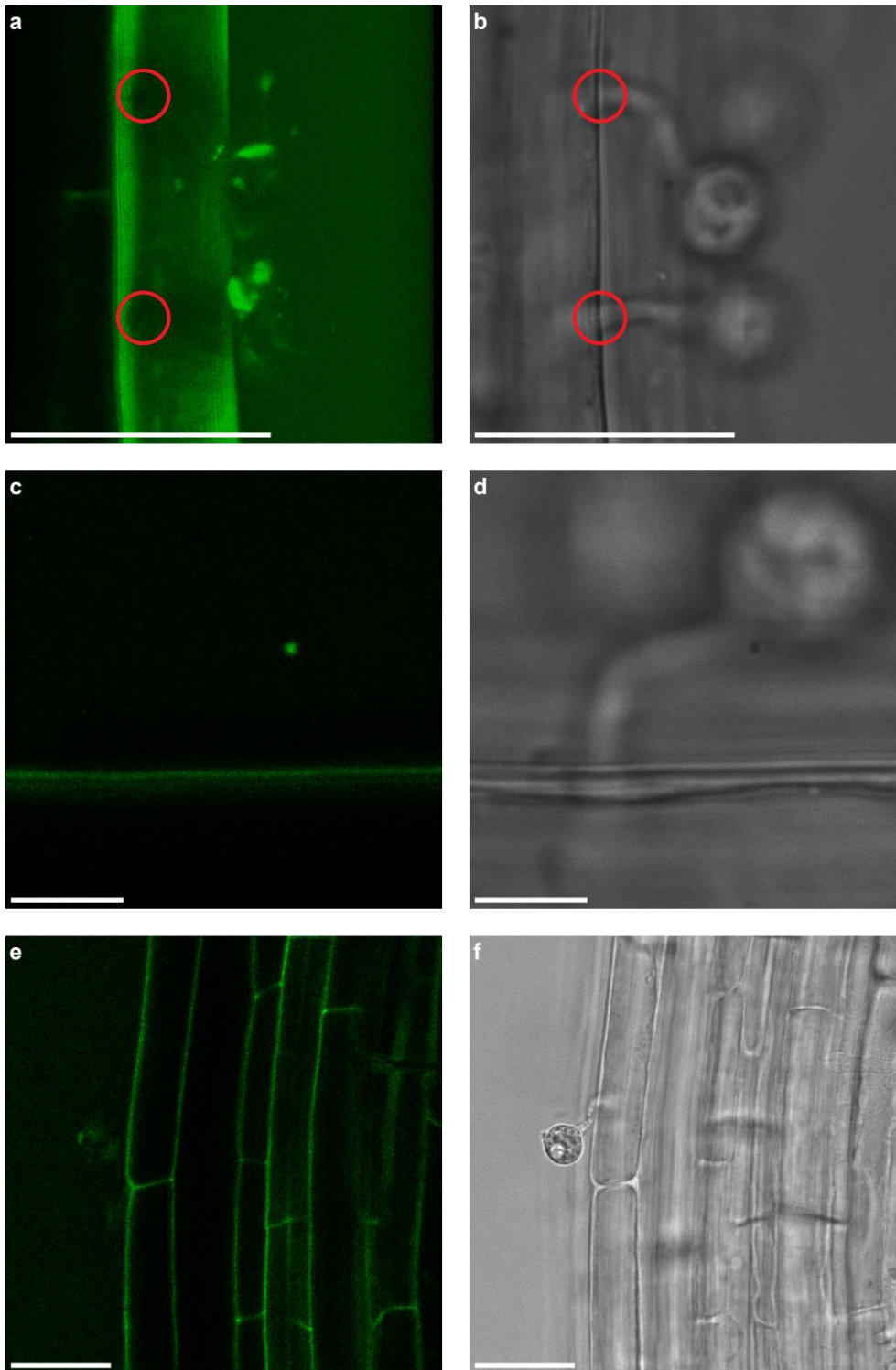


Figure S6: Fluorescence and corresponding brightfield images of *Arabidopsis* seedlings stained with 40 μ M CT-AF488 infected with *P. palmivora*. While the root cell wall is visible (a, c and d) and the site of penetration of the can be observed as a dark spot (a, red circles) no *Phytophthora* is observed in the fluorescence channel. Brightfield images (b, d and f) confirm the presence of *Phytophthora*. The image shown in panel a is a maximum intensity projection. Scale bars represent 25 μ m.

MICRORHEOLOGY

We, and others, have used the term microviscosity to describe the physical quantity that these probes are sensitive to [35, 60, 61]. However, in the context of the plant cell wall this term is misleading. The probe binds a cell wall epitope and sits in the water-filled meshes in-between biopolymer chains; the local aqueous viscosity in these pores will not change substantially when the cell wall composition and structure are altered. Rather, we believe that cell wall targeted BDP probes map cell wall porosity [34, 35]. The flow field caused by probe rotation will extend out to several times the size of the molecule, i.e. approximately several to ten nanometers. In larger pores, e.g. cellulose rich matured cell walls, the fluid can flow freely as it is not hindered by nearby polymer chains. In smaller pores, e.g. in pectin-rich young cell walls, the fluid flow is severely hindered by the network resulting in a slower rotation and hence a longer lifetime.

To test this hypothesis, we make use of the feature of polymer solutions that at the same mass concentration for a specific type of polymer, higher molecular weight polymers give a higher macroscopic solution viscosity but identical network mesh, or pore, size [62]. We measured the fluorescence lifetime of a sulfonated BDP rotor dissolved in solutions of the polymer poly(ethylene oxide) of different molecular weights and as a function of polymer concentration. For each sample, we independently measured the solution viscosity using light-scattering based microrheology [63, 64]. The BDP lifetime as a function of viscosity indeed does not yield a universal curve, indicating that the physical quantity that is measured is not the solution viscosity (Supplementary Fig. 5b). Rather, if we plot the fluorescence lifetime as a function of the mass concentration, we find a collapse of the data onto a single curve (Supplementary Fig. 5a); hence, the response is independent of polymer length and only of polymer concentration, i.e. mesh size of the polymer network. These results support the notion that BDP molecular rotors are cell wall porosity sensors.

We studied the fluorescence lifetime response of PEG-BDP in aqueous solutions of ethylene glycol, poly(ethylene glycol) of various chain lengths (Table S1) and poly(acrylic acid) 5.1 kg mol⁻¹. The concentrations were varied between 0.01 g cm⁻³ and 10 g cm⁻³ until reaching the solute solubility limit. The viscosity of those solutions was determined by DLS micro rheology.

Table S1: The average molecular weight and polydispersity (PDI) of PEG polymers used to study the response of PEG-BDP to viscosity and mesh size.

Polymer	M _n (g mol ⁻¹)	PDI
PEG1k	1000	<1.3
PEG10k	10000	<1.3
PEG100k	95000	1.08
PEG728k	728000	1.24

PMMA-PEGMA particles with a hydrodynamic radius of 77 nm (PDI between 0.002 and 0.042) were synthesized by a standard emulsion polymerization.

In a 250 mL round-bottom flask, methyl methacrylate (8.9 g) and PEG methacrylate (0.5 g) were mixed in MQ water (100 mL) containing a small amount of SDS (20 mg). The mixture was degassed by purging with nitrogen for 10 min. The flask was put under slight vacuum. The biphasic mixture was stirred at 75°C for 15min, and the stirring speed adjusted so as to avoid emulsification. A solution of potassium persulfate (100 mg) in MQ water (5 mL) was injected, and the reaction carried out for at least 24h. The obtained turbid suspension was filtered through glass wool and stored at 4°C in a plastic bottle.

The dynamic light-scattering experiments were carried out on a Malvern Nano-S, with a He-Ne laser ($\lambda=632.8$ nm), an avalanche photodetector at a detection angle of 173°. In all experiments the temperature was controlled at 20°C. The DLS particles were suspended in the samples of interest by diluting the stock 1000 times. The scattered intensity autocorrelation function was measured 3 times, for 1000 s to 1 h depending on the characteristic decorrelation time of each individual sample. The mean square displacement of the particles was determined from the scattered intensity autocorrelation functions, and the diffusion coefficient computed by using the slope of the mean square displacement vs time plot at time scales beyond the caging dynamics. The diffusion coefficient was directly translated into a viscosity using the Stokes-Einstein equation.

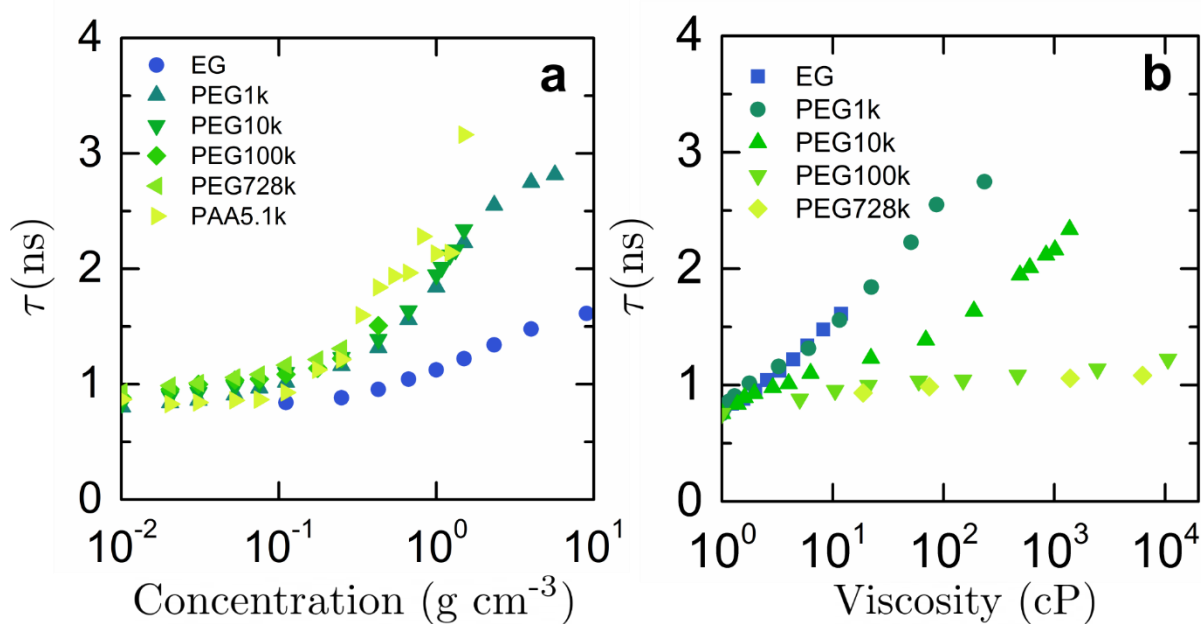


Figure S7: Evolution of the fluorescence lifetime of PEG-BDP in solutions of EG, PEG and PAA with various chain length, as a function of EG or polymer concentration (a), and as a function of viscosity as measured by DLS microrheology (b)

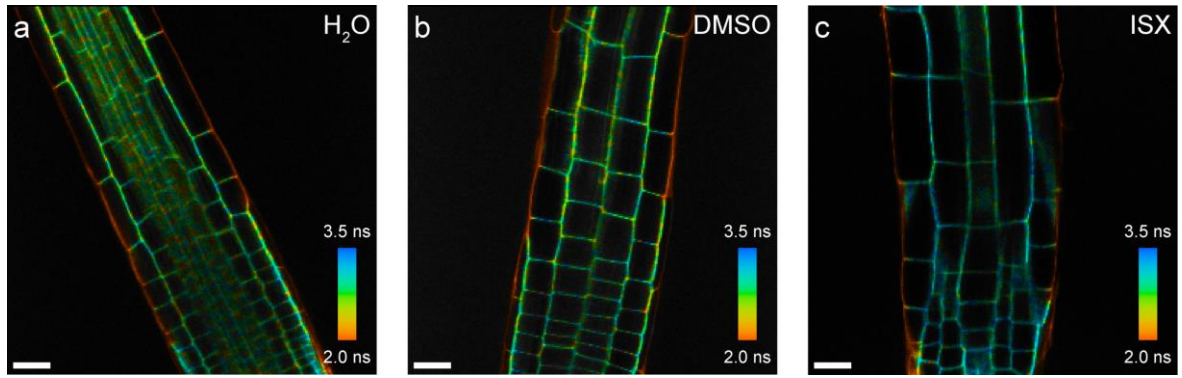


Figure S8: FLIM images of non-treated (a), mock treated (b) and isoxaben (ISX) treated (c) Col-0 incubated with CT-BDP. Scale bars represent 25 μm .

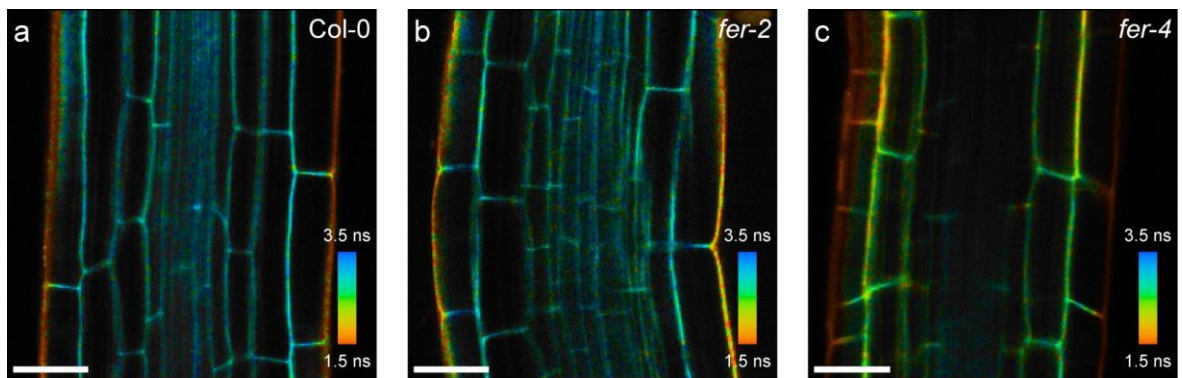


Figure S9: FLIM images of Col-0 (a), *fer-2* (b) and *fer-4* (c) mutants incubated with CT-BDP. Scale bars represent 25 μm .

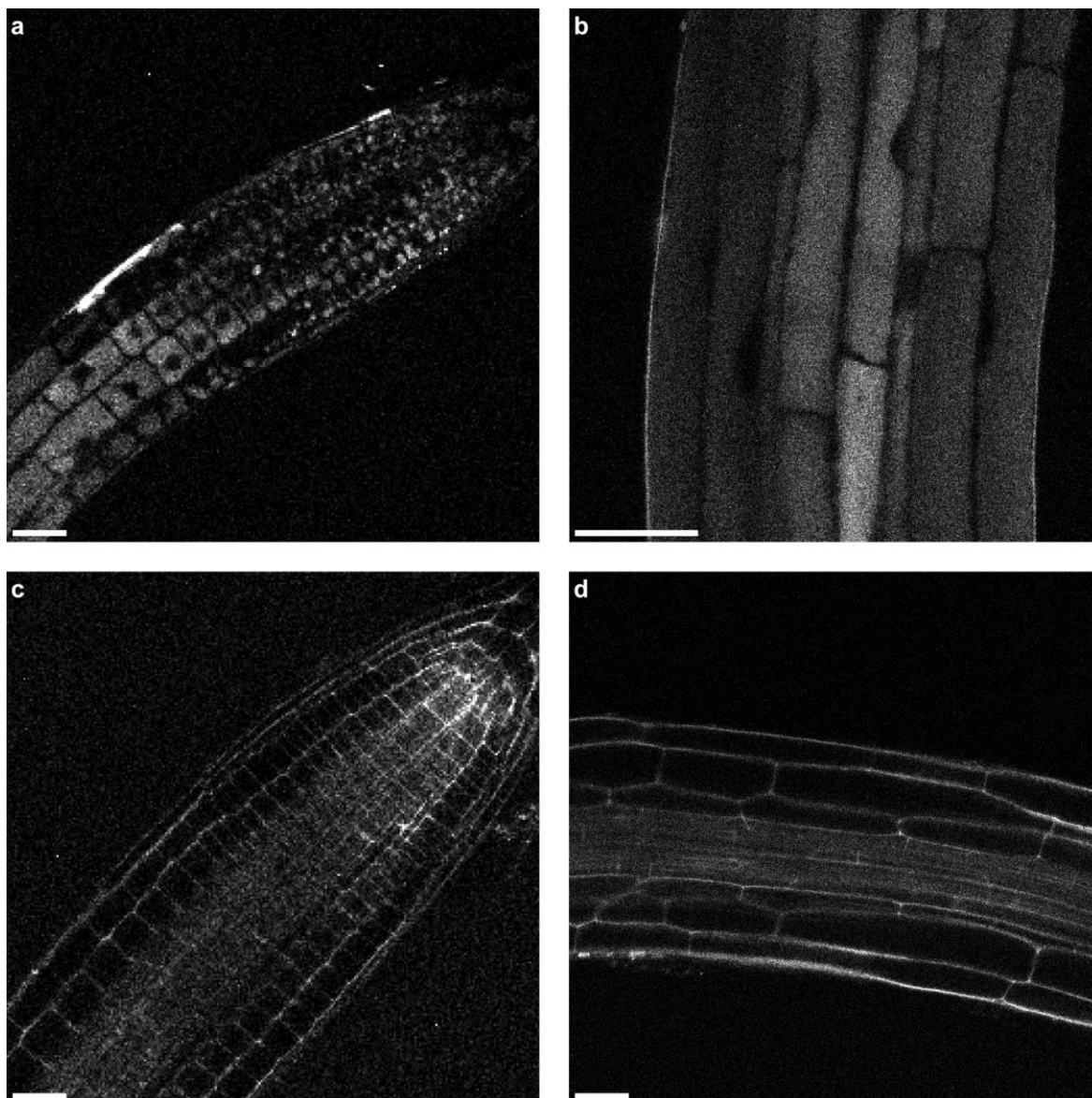


Figure S10: a-b) Arabidopsis roots stained with 10 μM (a) and 40 μM (b) non-functionalized Oregon Green from a 5 mg/ml stock in DMF exhibit no cell wall staining but do exhibit internalization after 1 h. c-d) Arabidopsis roots stained with 10 μM (c) and 40 μM (d) CarboTag modified Oregon Green imaged under imaging conditions compatible with FLIM exhibit cell wall specificity. Scale bars represent 25 μm .

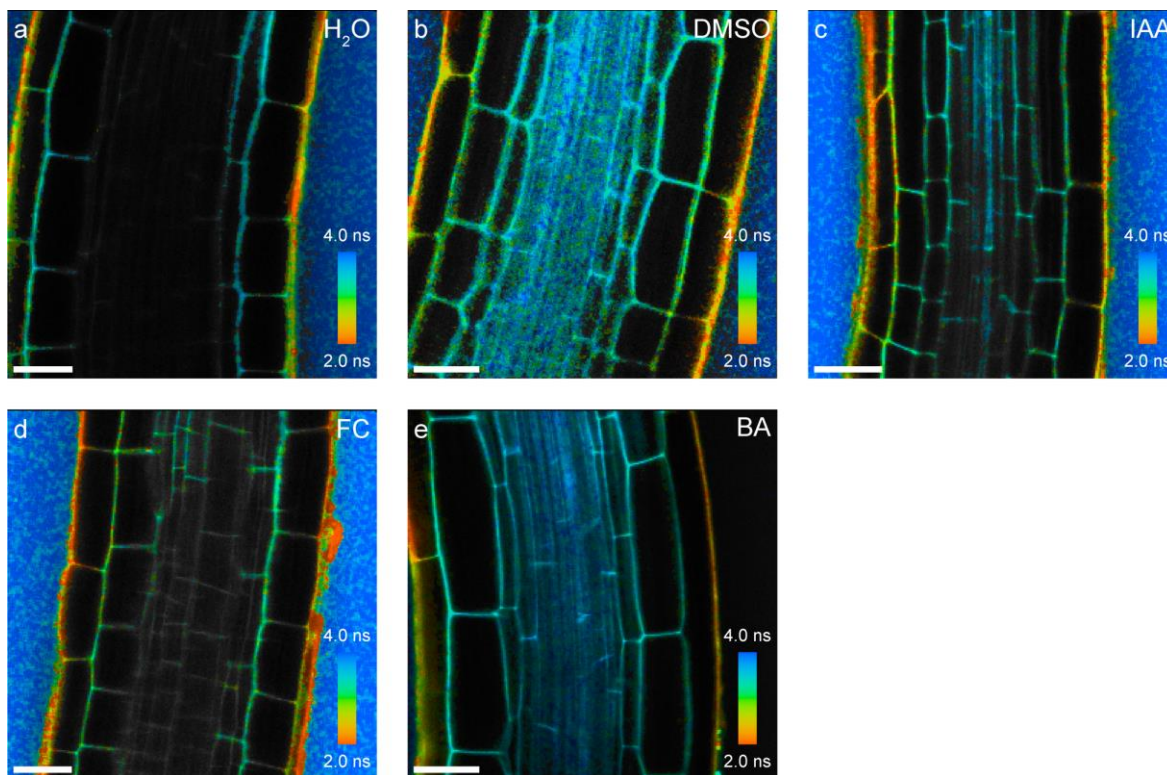


Figure S11: FLIM images of roots incubated with 40 μM CT-OG after 10 minutes of no treatment (H₂O, a), mock treatment (DMSO, b), 1 μM auxin (IAA, c), 10 μM fusicoccin (FC, d) and 1 μM benzoic acid (BA, e). Scale bars represent 25 μm

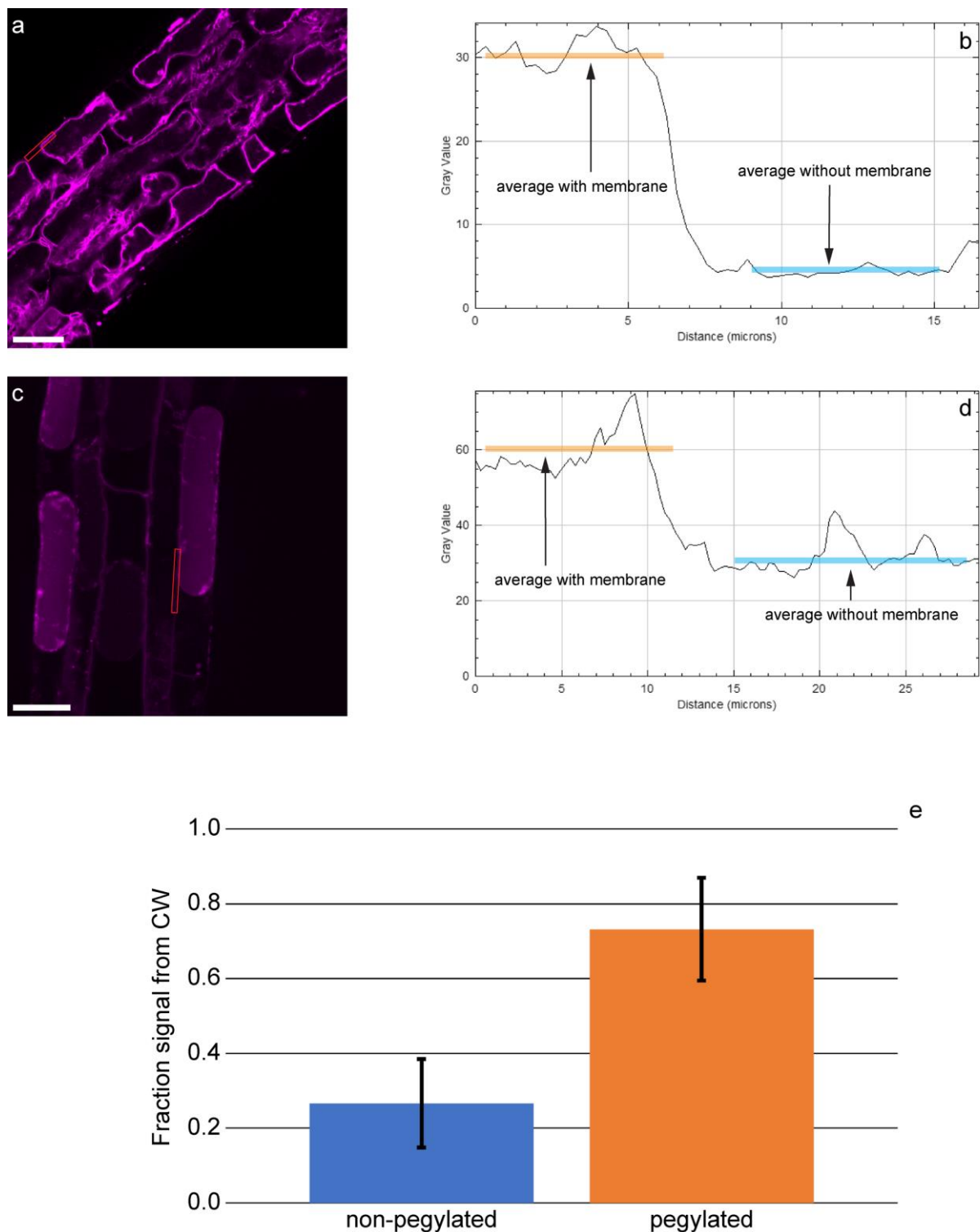


Figure S12: plasmolysis of non-pegylated CarboTag ROS probe (a) and pegylated CarboTag-Ox (c) shows high membrane affinity for the CarboTag ROS probe and limited membrane localization for CarboTag-Ox. CarboTag-Ox however internalizes which could potentially be caused by a pegylated intermediate present in the staining solution. Using an ROI following the cell wall and membrane (in red) we determined the signal originating from the cell wall + membrane and only the cell wall for CarboTag ROS (b) and CarboTag-Ox (d). The fraction of the signal originating from the cell wall was calculated by dividing the average intensity in the cell wall by the average intensity of the area with both the cell wall and cell membrane (e), $n=3$ for the non-pegylated and $n=4$ for the pegylated probe. Scale bars represent 25 μm .

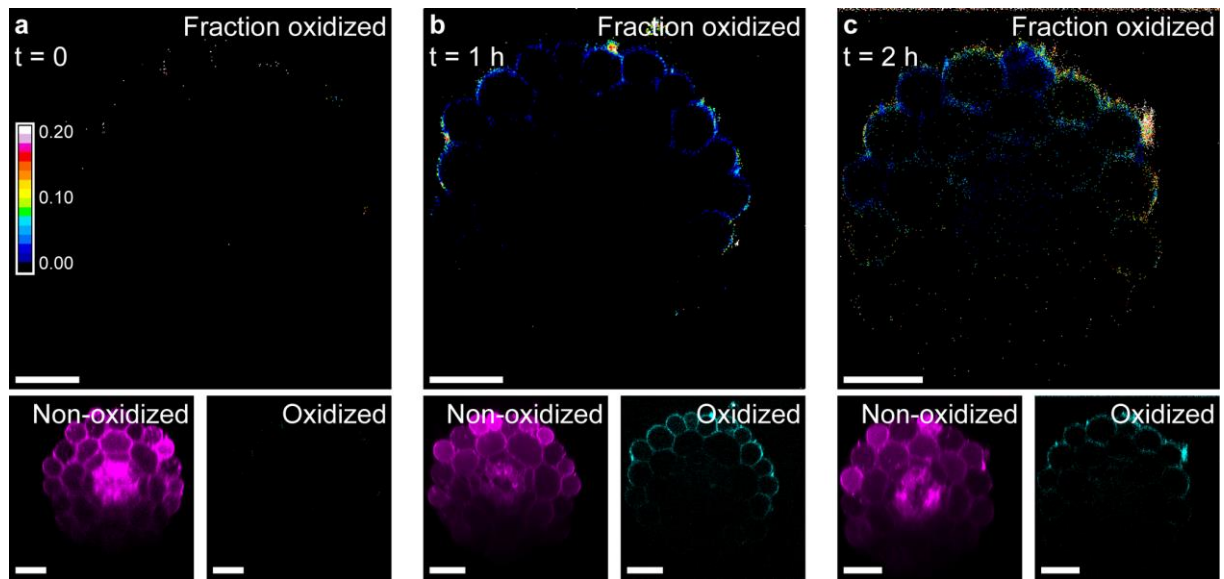


Figure S13: Timelapse of CarboTag-Ox ROS probe stained Arabidopsis roots vacuum treated for 5 minutes without flg22. Color corresponds to the fraction of oxidized probe of the total fluorescence signal (oxidized + non-oxidized). Non-oxidized and oxidized signal are shown separately in magenta and cyan respectively. Scale bars represent and 25 μm .

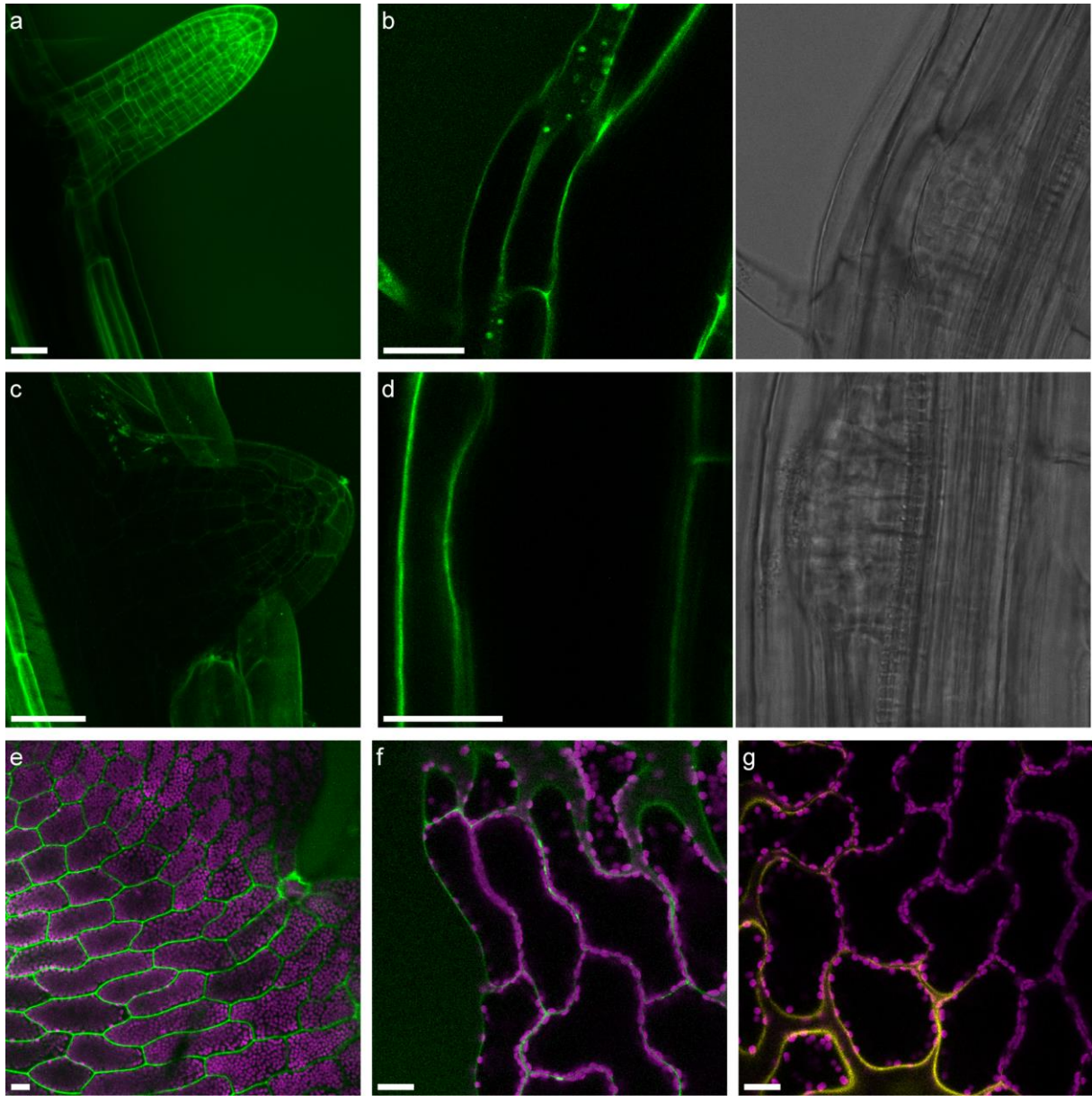
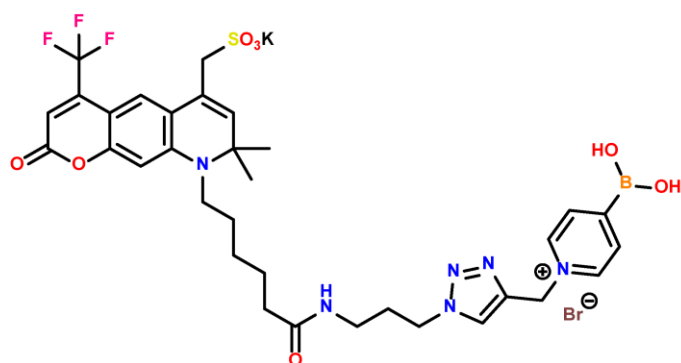
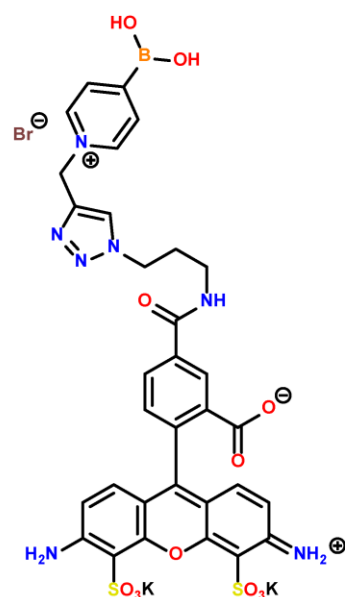


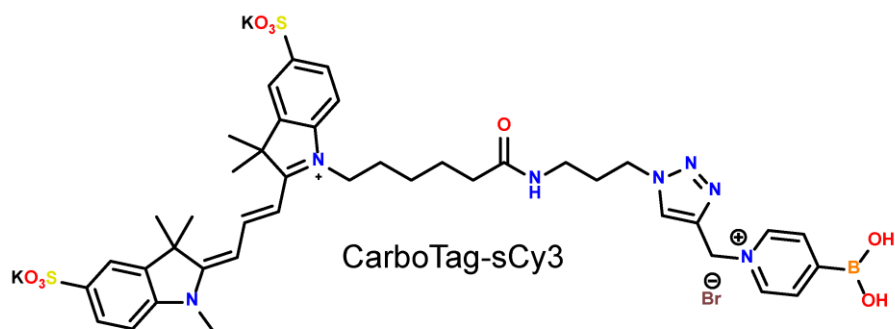
Figure S14: a) Average intensity projection of an emerged lateral root stained with CT-AF488 shows good staining. Internal lateral root primordia (b,d) and recently emerged lateral root (c) show no or limited staining. e) Maximum intensity projection of a fern thallus stained with CT-AF488 shows decent staining but is difficult to replicate. f,g) fern thalli stained with CT-AF488 (f) and CT-sCy3 (g) show poor staining even after 6 h. Scale bars represent 25 μm .



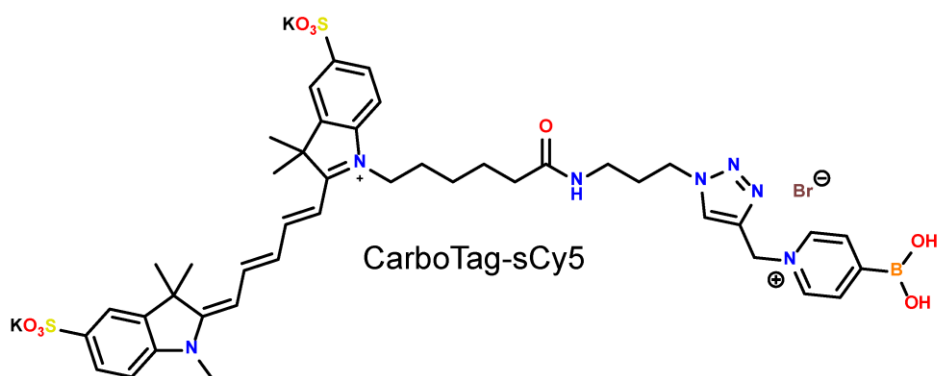
CarboTag-AF430



CarboTag-AF488



CarboTag-sCy3



CarboTag-sCy5

Figure S35: chemical structures of commercially available azide functionalized fluorophores modified with CarboTag.

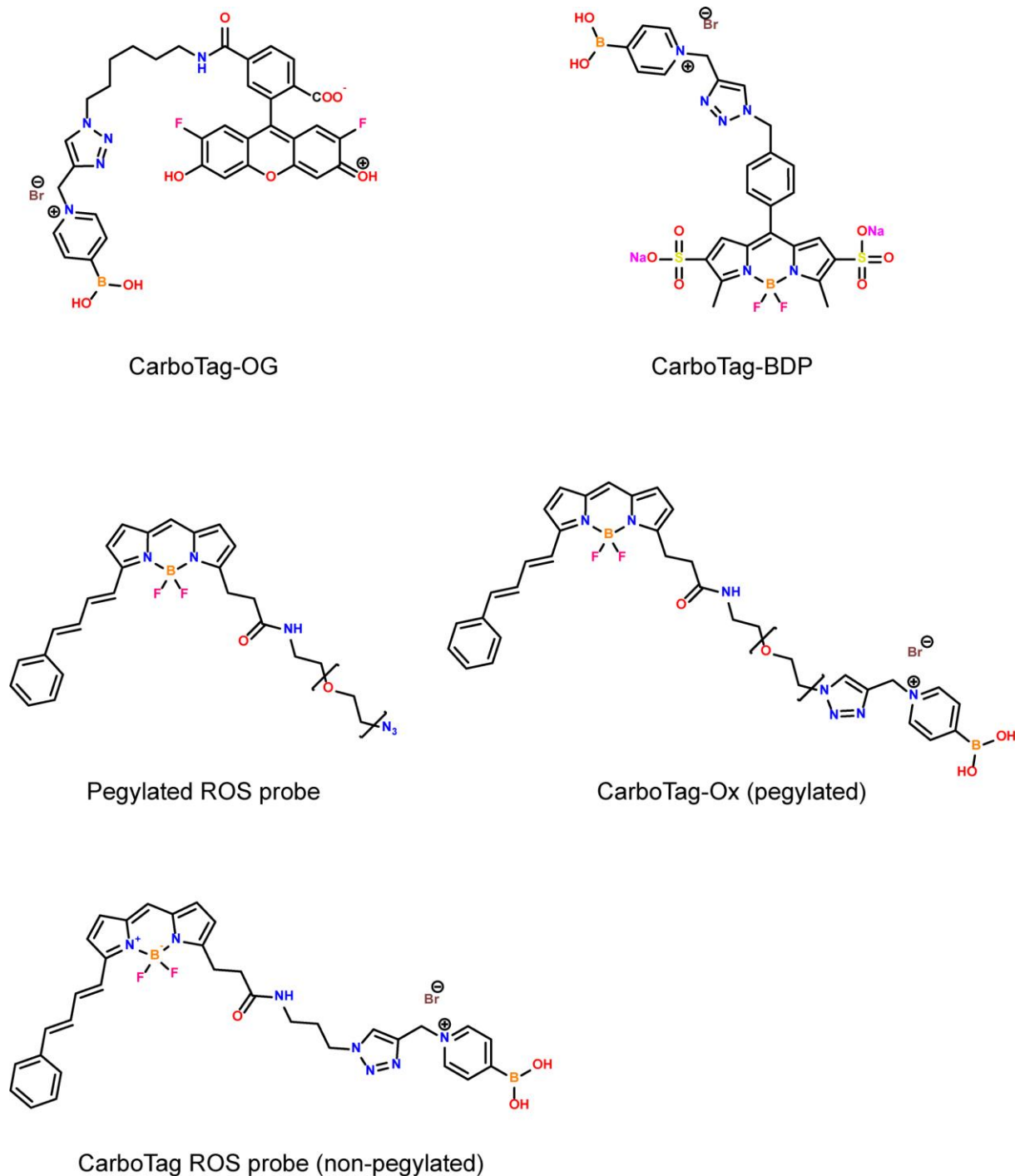


Figure S16: chemical structures of pH (CarboTag-OG), porosity (CarboTag-BDP) and ROS (CarboTag-Ox) probes. A fraction of pegylated precursor of CarboTag-Ox (pegylated ROS probe) is likely to be present the isolated CarboTag-Ox. Similar pegylated probes have shown to internalize in the vacuole and cytoplasm over time which explains why there is some internalization of CarboTag-Ox in root cells. The membrane localizing CarboTag ROS probe lacking a PEG-8 moiety is shown in the bottom.

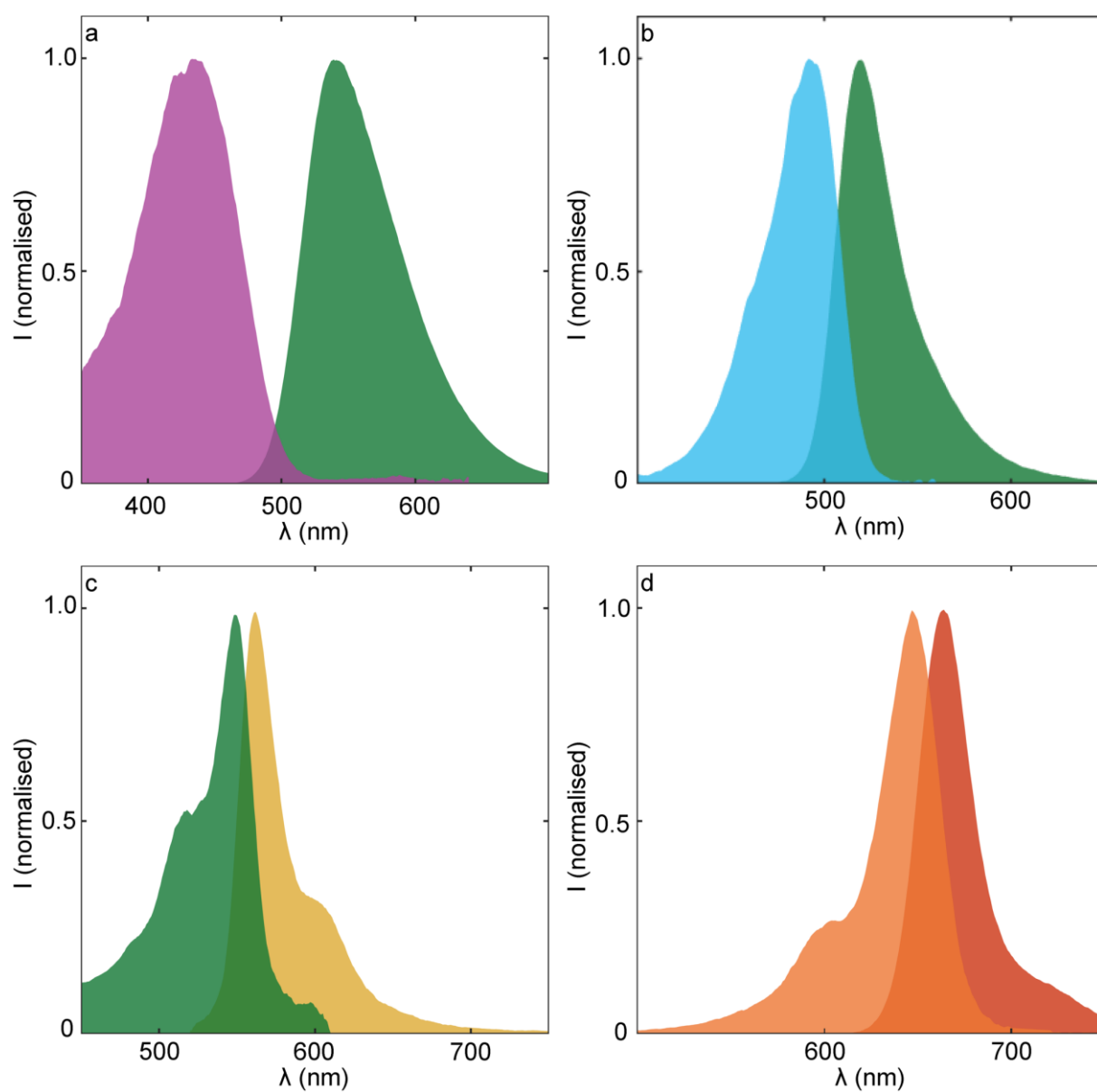


Figure S47: excitation emission spectra of CT-AF430 ($\lambda_{ex} = 430$ nm, $\lambda_{em} = 650$ nm), CT-AF488 ($\lambda_{ex} = 450$ nm, $\lambda_{em} = 570$ nm), CT-sCy3 ($\lambda_{ex} = 510$ nm, $\lambda_{em} = 620$ nm) and CT-sCy5 ($\lambda_{ex} = 600$ nm, $\lambda_{em} = 710$ nm).

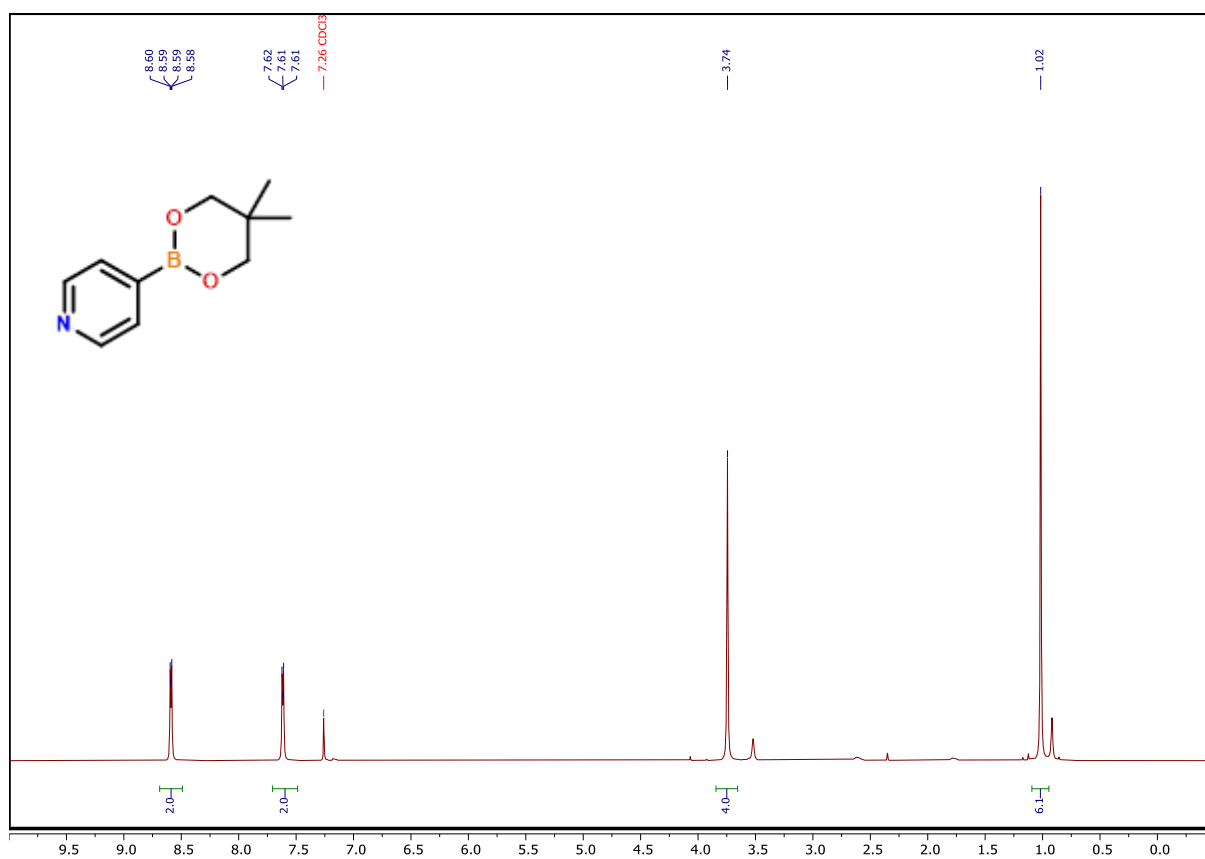


Figure S18: ^1H NMR spectrum of **1**

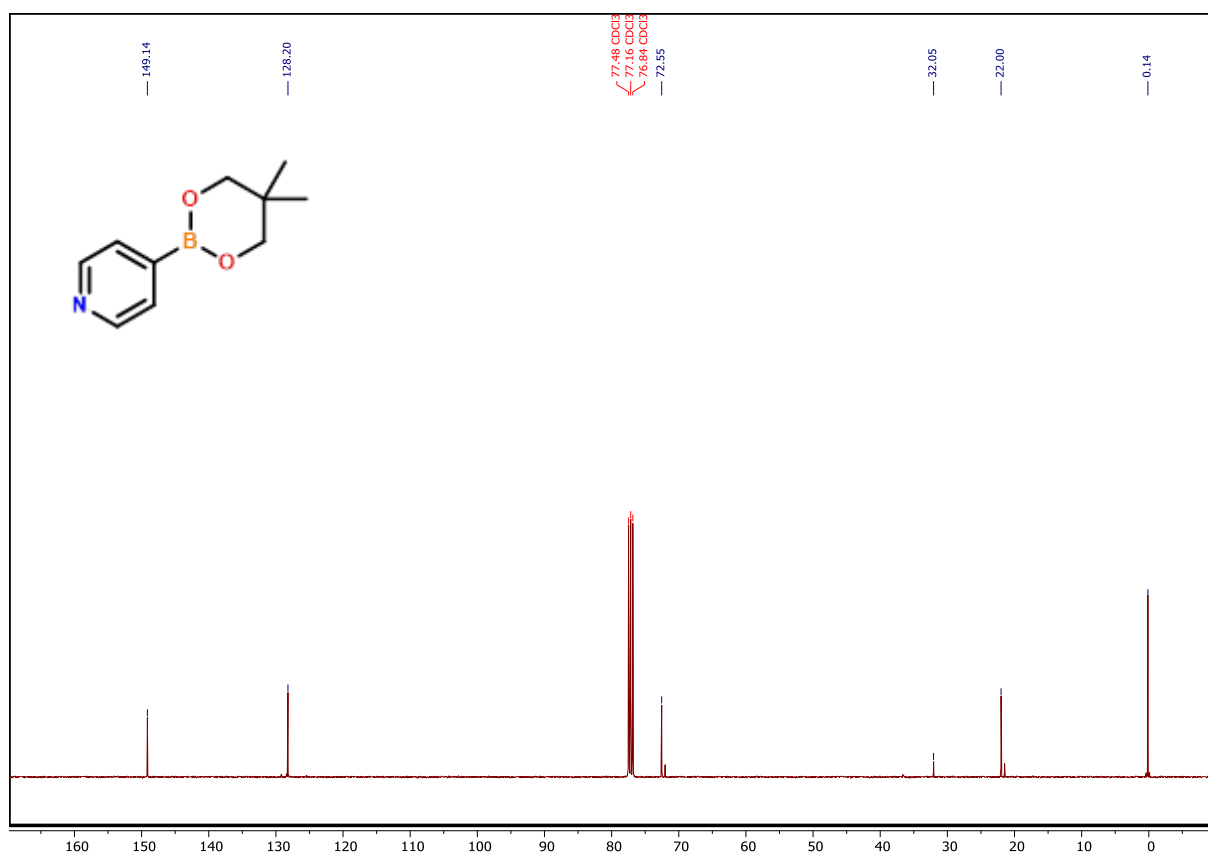


Figure S19: ^{13}C NMR spectrum of **1**

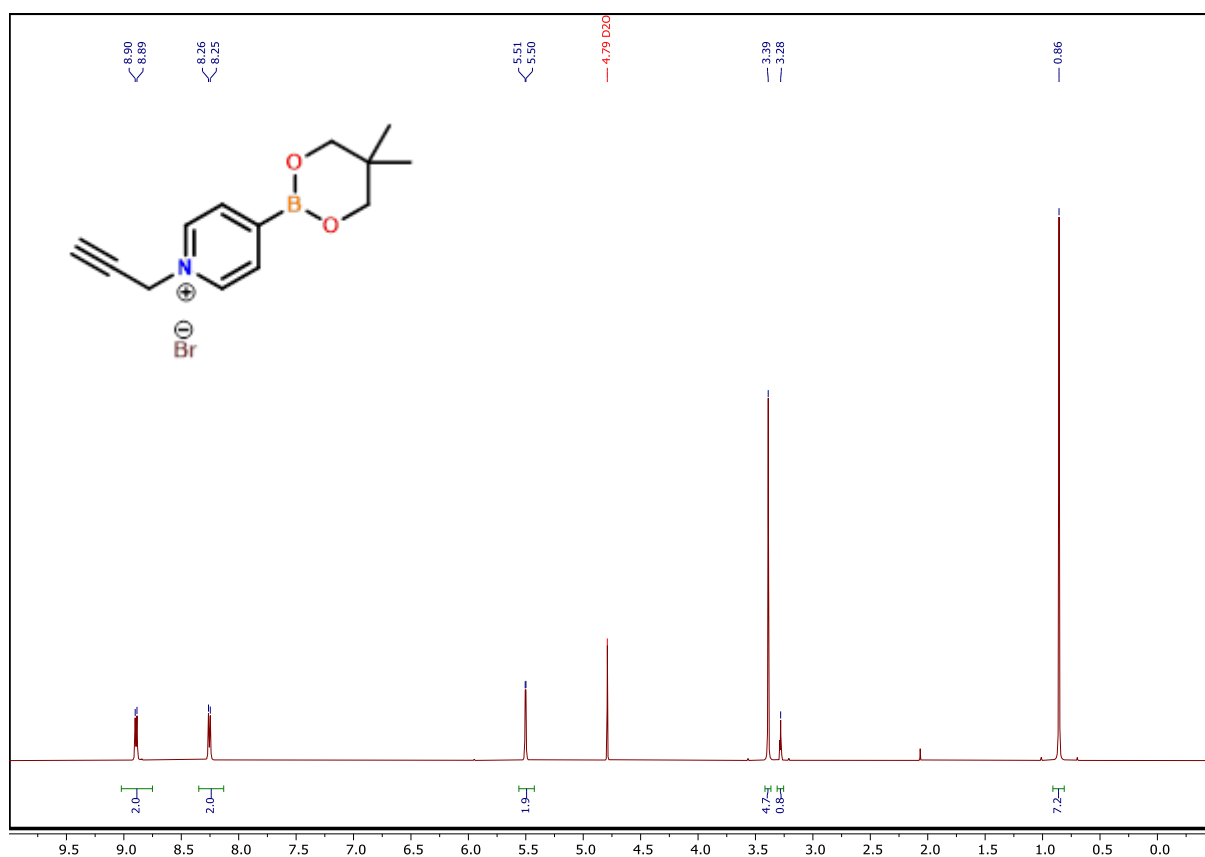


Figure S50: ¹H NMR spectrum of **2**

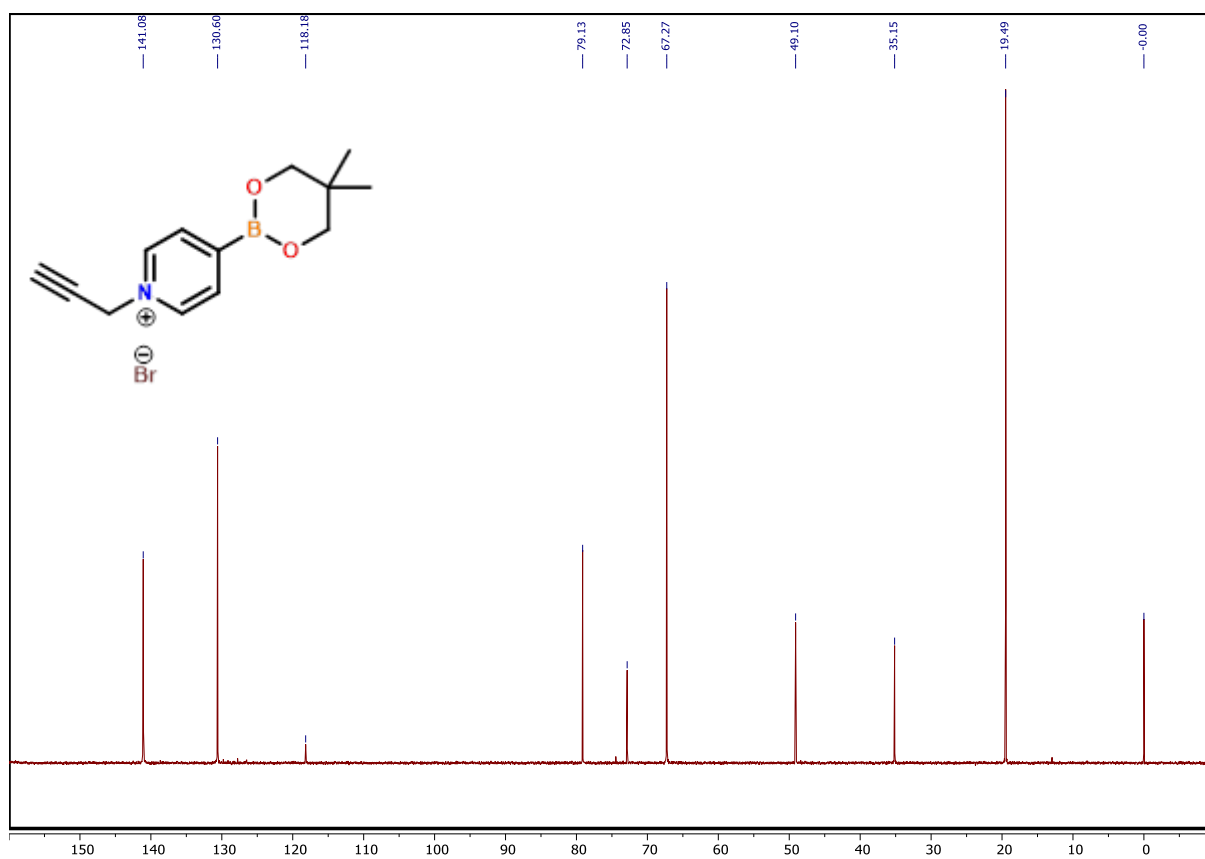


Figure S61: ¹³C NMR spectrum of **2**

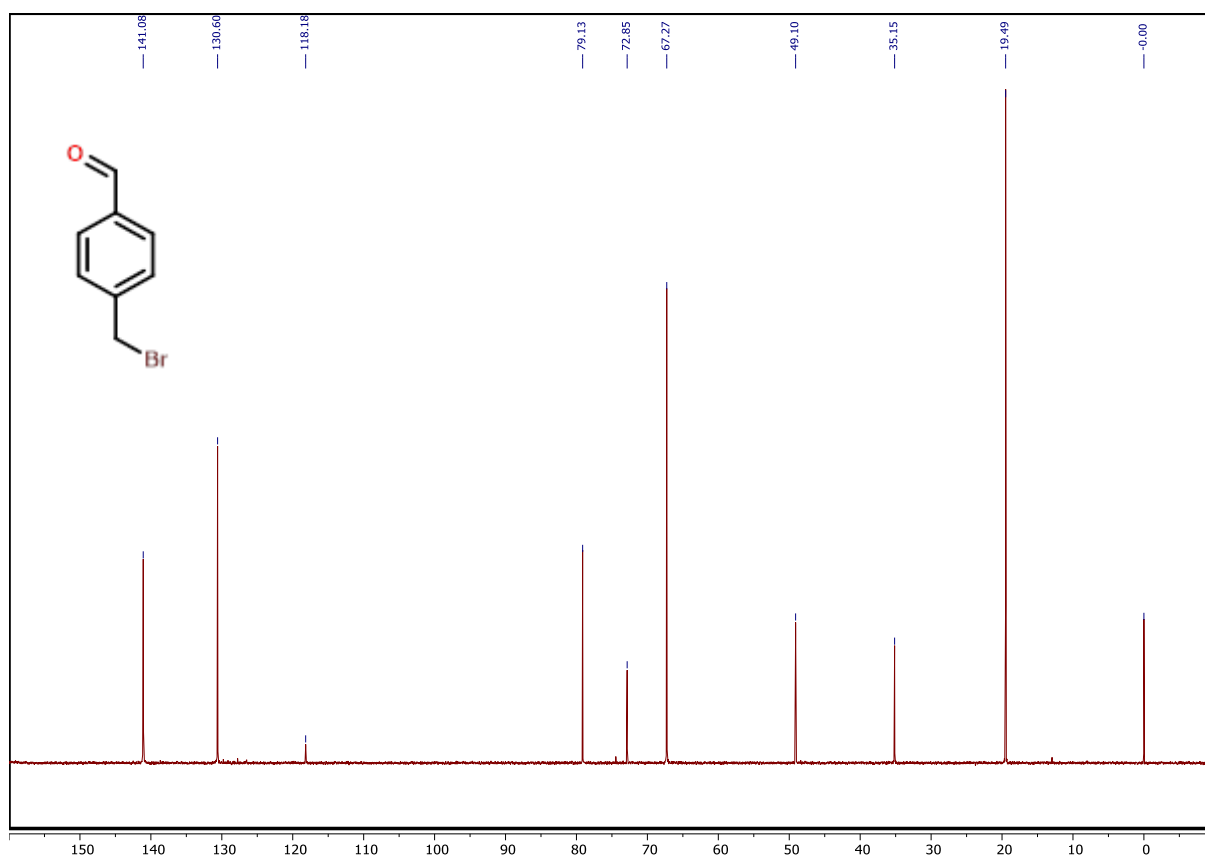


Figure S72: ^1H NMR spectrum of **3**

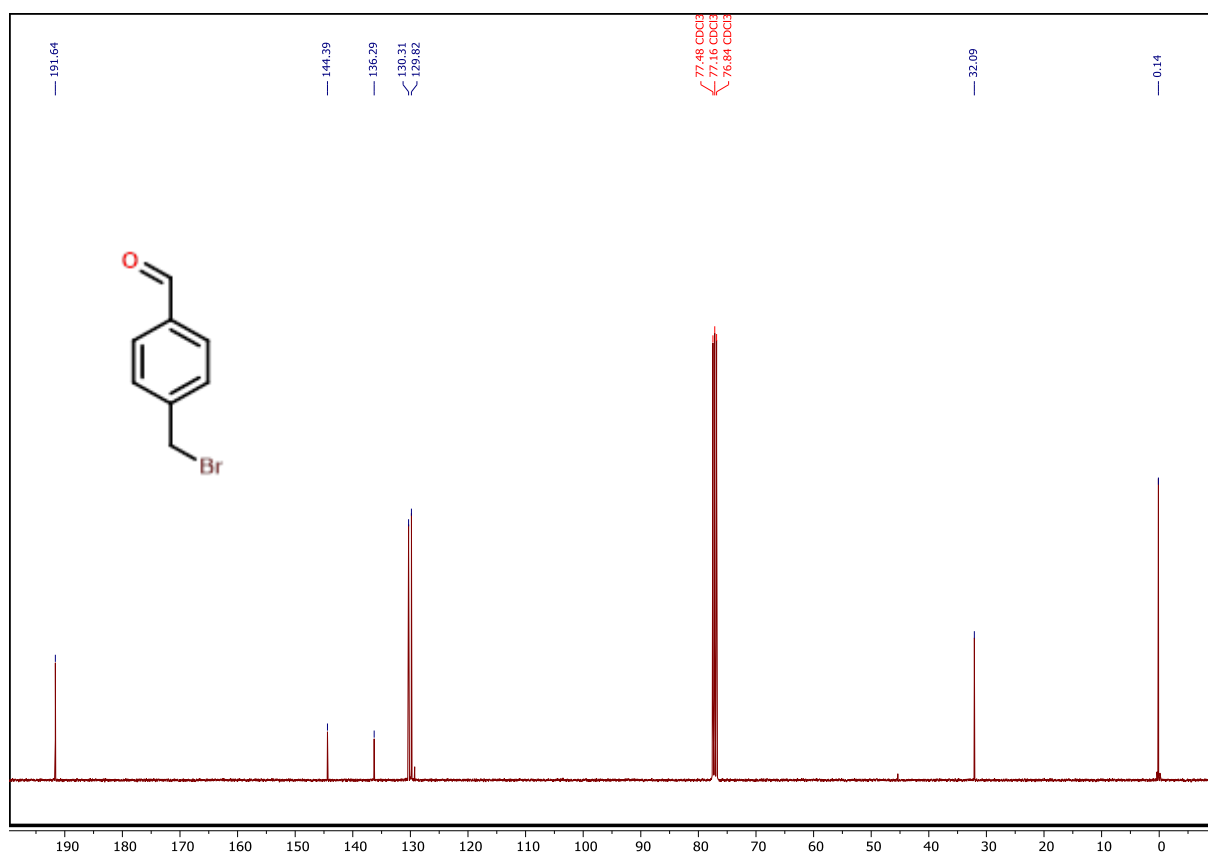


Figure S83: ^{13}C NMR spectrum of **3**

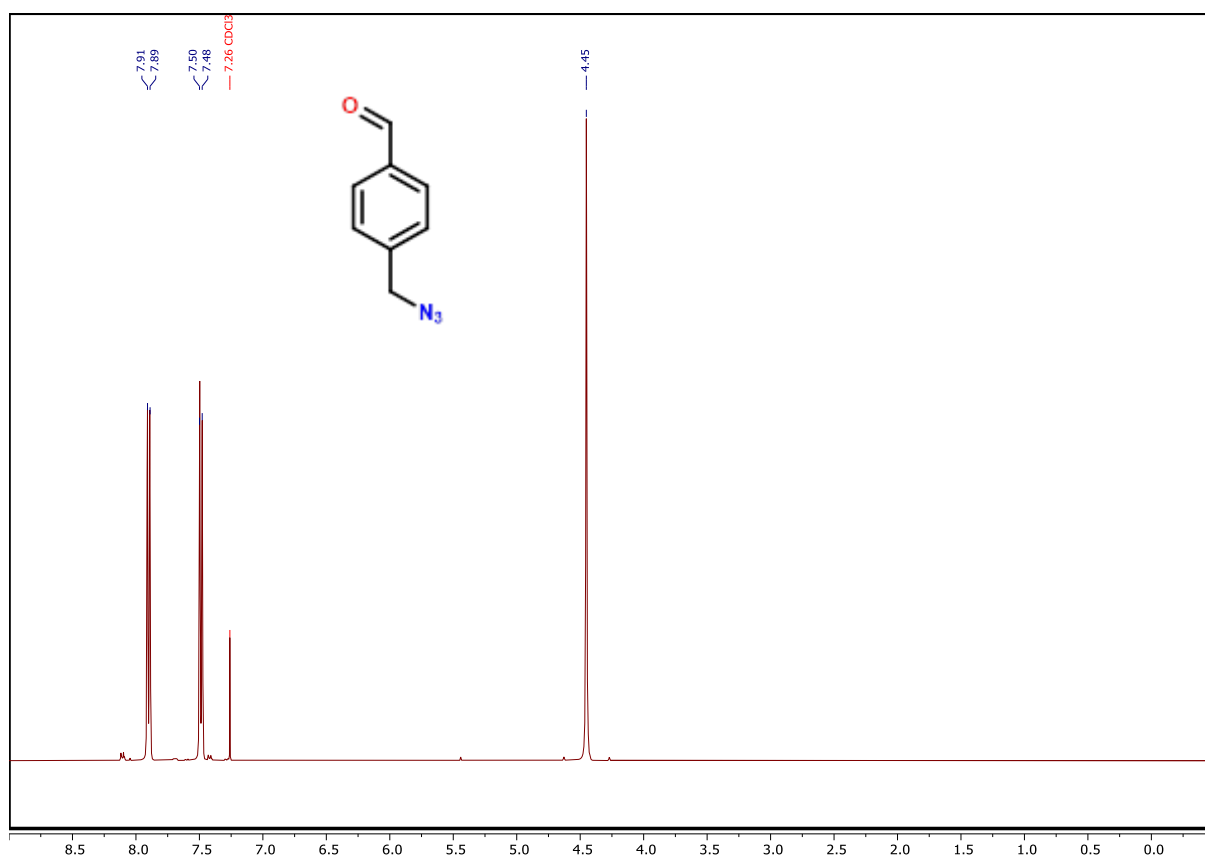


Figure S94: ^1H NMR spectrum of **4**

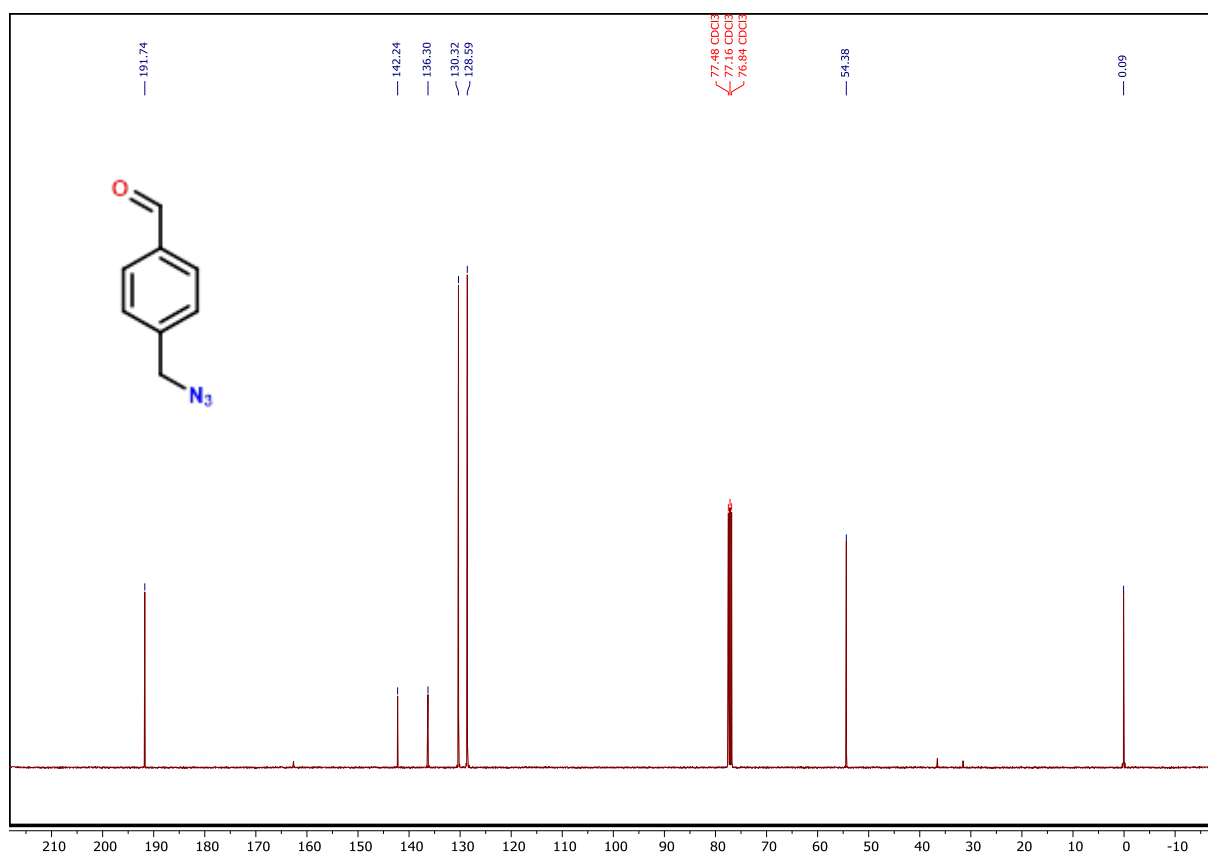


Figure S105: ^{13}C NMR spectrum of **4**

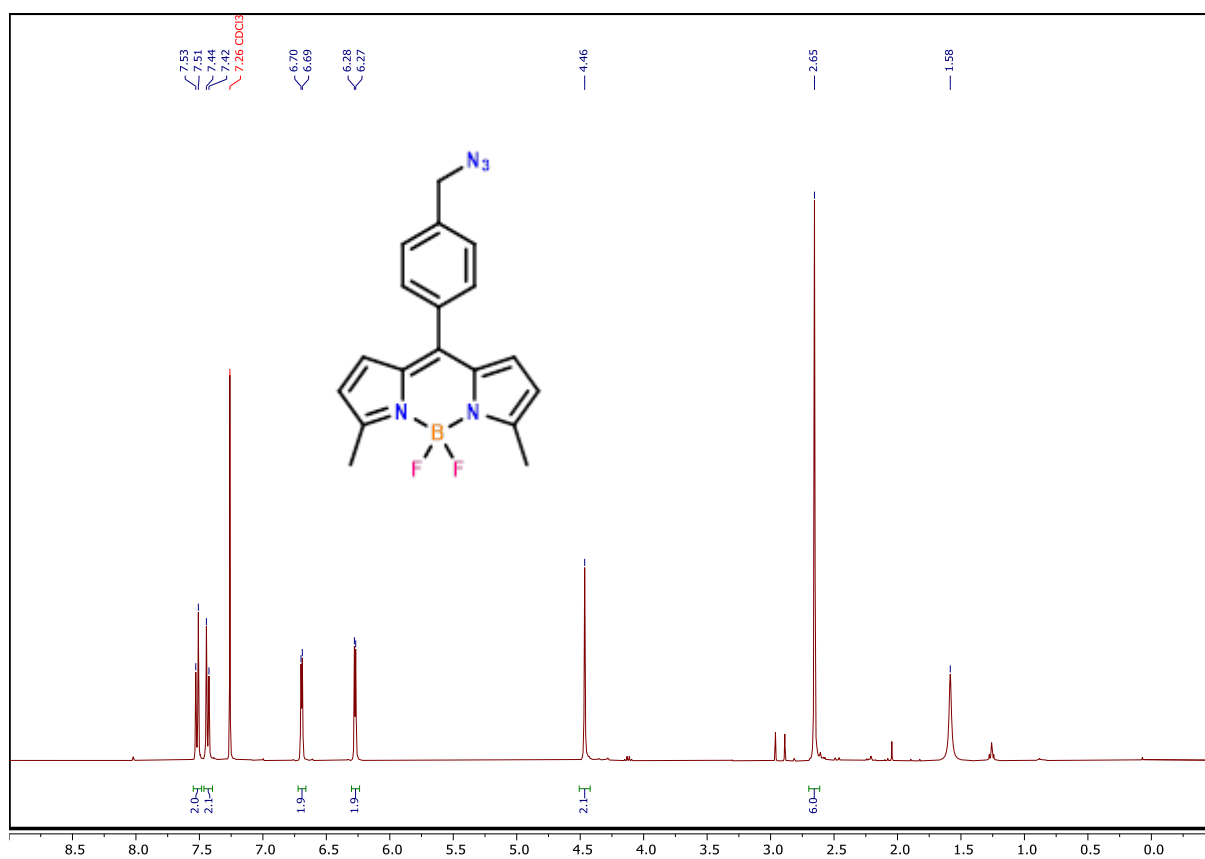


Figure S116: ^1H NMR spectrum of **5**

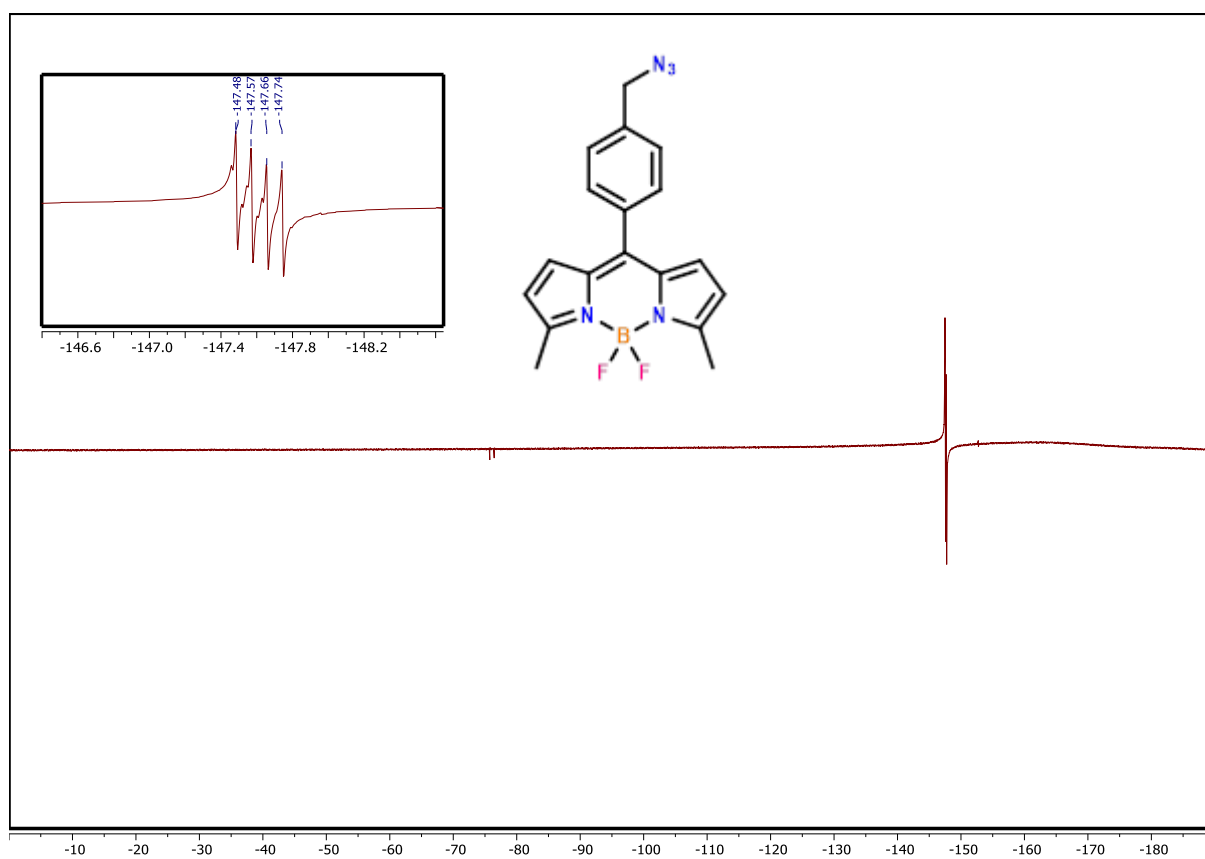


Figure S127: ^{19}F NMR spectrum of **5**

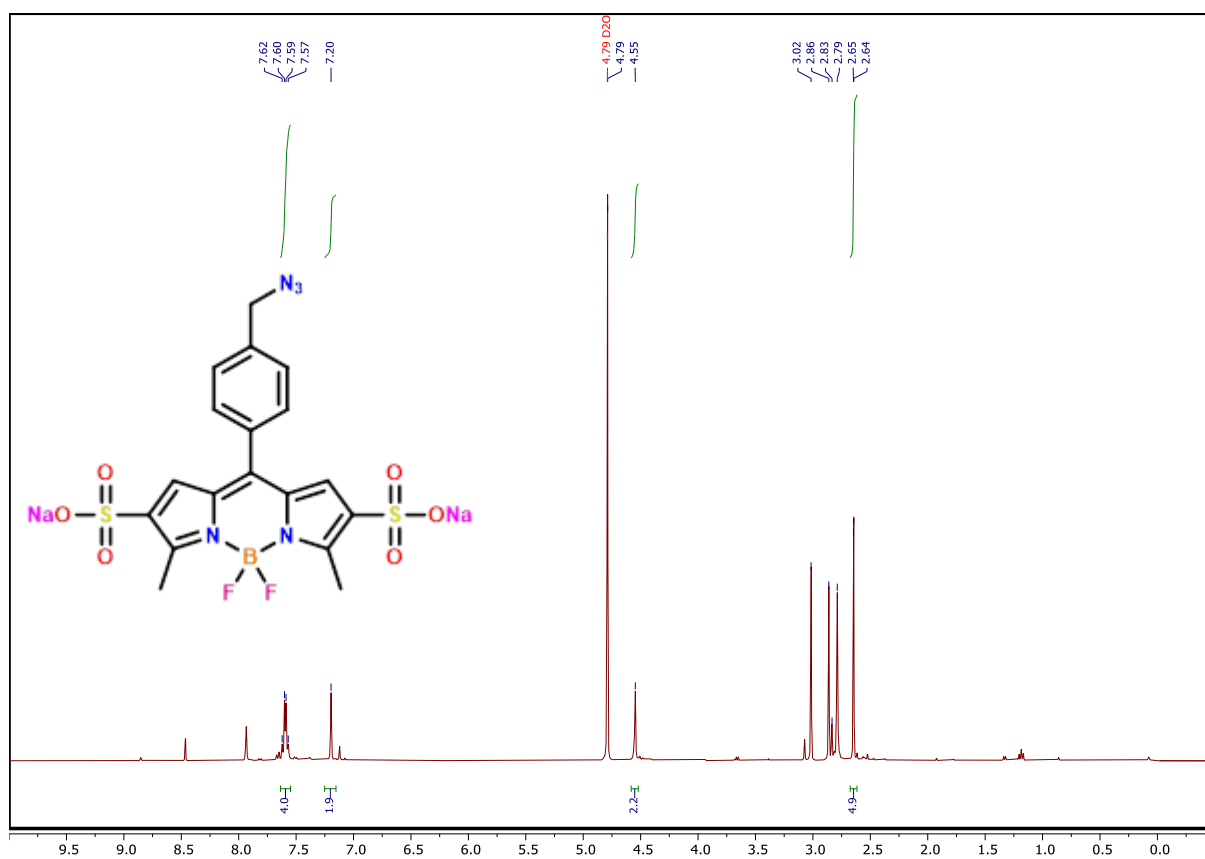


Figure S138: ^1H NMR spectrum of **6**