

Electronic Supporting Information of

Far Red and NIR Dye-Peptoid Conjugates for Efficient Immune Cell Labelling and Tracking in Preclinical Models

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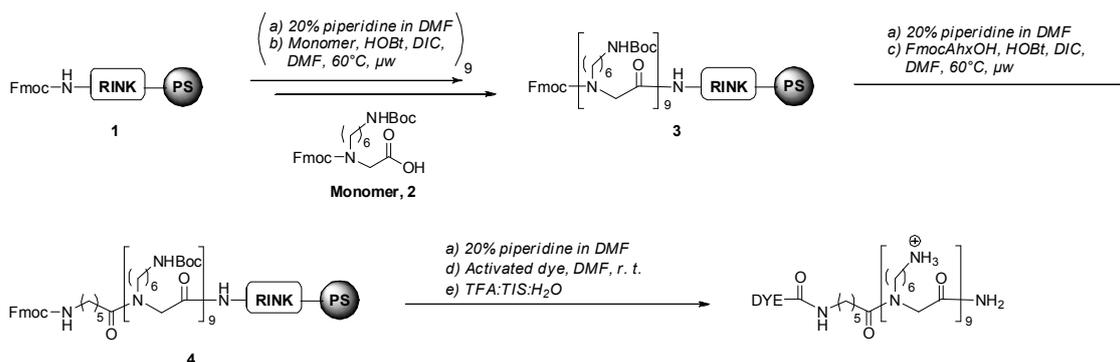
1. Synthesis and characterisation of the dye-peptoids

1.1. General information

All solvents and reagents were obtained from commercial sources and used without further purification unless otherwise noted. The presence of free primary amines was determined by a conventional ninhydrin test. The presence of free secondary amines was determined by a chloranil test. ^1H spectra were recorded on a Bruker DMX500 spectrometer in D_2O at 298K. ES/MS (low resolution electrospray mass spectra) were recorded on an Agilent Technologies LC/MSD 1100 Quadrupole Mass Spectrometer (QMS) with an electrospray ion source. MALDI (Matrix Assisted Laser Desorption) experiments were performed using sinapic acid as a matrix using a Voyager-DETM STR. Microwave reactions were carried out using a Biotage Initiator instrument and in sealed heavy-walled Pyrex tubes. HPLC and ELSD analyses were performed on an Agilent 1100 analytical system with a Supelco Discovery[®] C18, 5 μm , 5 cm column coupled to a Polymer laboratories 100 ES evaporative light scattering detector. UV/VIS analyses were performed on an Agilent 8453 spectrophotometer using a MeOH blank.

1.2. Solid-Phase Synthesis of dye-peptoids

Carboxy-containing Cy5.5 and Cy7 fluorophores¹ and the peptoid monomer² were synthesized as previously described. Cell penetrating peptoids were synthesized using a highly-optimised microwave-based solid-phase strategy as illustrated in the Scheme S1. Peptoids were assembled on aminomethyl polystyrene resin functionalized with a Rink amide linker, **1**, using an Fmoc-based strategy and monomer **2**.² Peptoid assembly was carried out using a two-step procedure: (i) firstly, piperidine-mediated Fmoc deprotection; (ii) secondly, coupling with monomer **2** using HOBt/DIC as coupling reagents and microwave irradiation.³ This procedure was repeated nine times to give the oligomer of the desired length. The resulting resin **3** was Fmoc deprotected and coupled to Fmoc-Ahx-OH using the same procedure as before to give resin **4**. This was coupled to the required dye, and finally cleaved from the resin to give the dye-peptoids.



Scheme S1. General synthesis of the fluorescently-labelled 9-mer peptoids.

Rink amide coupling: Fmoc-linker (3 equiv.), DIC (3 equiv.) and HOBt (3 equiv.) in DMF at a concentration of 0.2 M for 4 hours.

Deprotection protocol: 20 % piperidine in DMF. 2 times x 10 min.

Monomer coupling protocol: monomer **2** (3 equiv.), DIC (3 equiv.) and HOBt (3 equiv.) in DMF at a concentration of 0.2 M were mixed and shaken for 15 minutes and the resulting solution was added to the resin and microwave irradiated at 60 °C for 20 min.

Coupling to FmocAhxOH: 6-(Fmoc-amino)hexanoic acid was coupled to resin **3** using the above coupling procedure.

Cy5.5 coupling: Carboxy-Cy5.5¹ was activated using a mixture of DIC (2 equiv.), *N*-hydroxysuccinimide (2 equiv.) and DMAP (0.2 equiv.) in DMF at a concentration of 0.2 M at

overnight. The solution containing the NHS-activated Cy5.5 was added to resin **4** and stirred for 4h. The mixture was protected from the light by aluminium foil. The coupling was monitored by a ninhydrin test until completion.

Cy7 coupling: Carboxy-Cy7¹ was activated using *N-N'*-disuccinimidylcarbonate (1.5 equiv.) in a solution of anhydrous DMF:pyridine (1:1) at 35°C for 3 hours under nitrogen. The reaction was monitored by HPLC and MS until completion. Subsequently, the solvent was evaporated under vacuum and the residue was precipitated in cold diethyl ether and dried overnight in a vacuum oven. A solution of NHS-activated Cy7 in anhydrous DMF (0.2 M) was then added to resin **4** and the mixture stirred overnight at room temperature, covered with aluminium foil to protect the dye. Coupling completion was monitored by a ninhydrin test.

Cleavage protocol: Resin-bound labelled peptoids were deprotected and cleaved from the solid support by treatment with a mixture of TFA:TIS:H₂O (95:2.5:2.5) for 4 h. The solvent was removed under vacuum; resulting solids were then dissolved in a small volume of TFA and precipitated in cold diethyl ether to give the TFA salts of Cy5.5-9mer, **5**, and Cy7-9mer, **6**, as blue solids.

1.3. Characterisation of Dye-peptoids

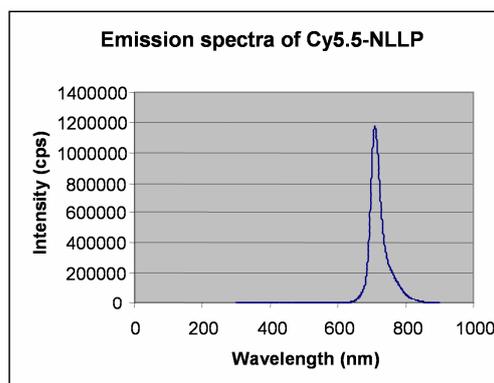
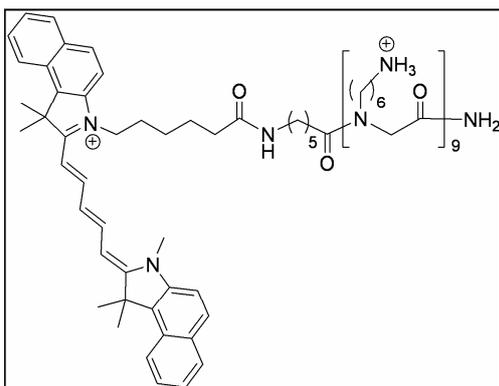
Cy5.5-9mer (5):

MALDI: $C_{118}H_{199}N_{22}O_{11}^+$ (M^+): calcd 2102.98, found 2102.28

m/z (ES^+): 700.26 ($[M+3]^{3+}$), 526.0 ($[M+4]^{4+}$), 421.0 ($[M+5]^{5+}$), 351.3 ($[M+6]^{6+}$)

HPLC: t_R : 4.051 min, purity > 97% (ELSD 675 nm).

Absorption max 623 nm Emmission max 710 nm



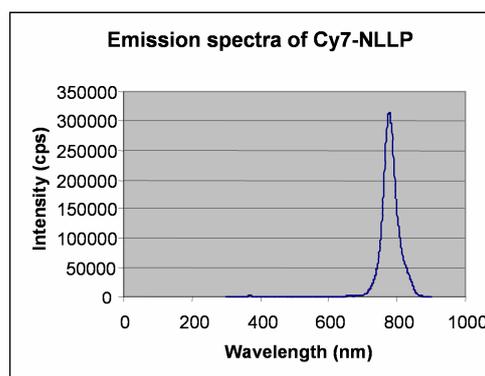
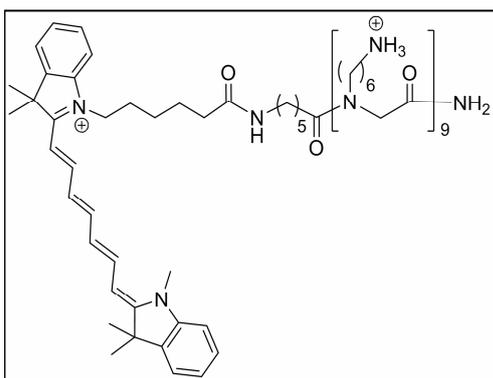
Cy7-9mer (6):

MALDI: $C_{112}H_{197}N_{22}O_{11}^+$ (M^+): 2026.55 calcd, found 2026.19.

m/z (ES^+): 507.5 ($[M+4]^{4+}$), 406.2 ($[M+5]^{5+}$), 338.7 ($[M+6]^{6+}$), 290.4 ($[M+7]^{7+}$)

HPLC: t_R : 4.85 min, purity >98% (ELSD 743nm).

Absorption max 693 nm Emmission max 779 nm



References:

- 1- Lopalco, M. et al. (2009) *Org. Biomol. Chem.* 7, 856-859.
- 2- Unciti-Broceta, A. et al. (2009) *Bioorg. Med. Chem.* 17, 959-966.
- 3- Fara, M. A. et al. (2006) *Tetrahedron Lett.* 47, 1011-1014.

2. Biological *in vitro* assays

2.1. Cell retrieval and viability assays

All animal procedures were performed with ethical review and licences. CD1 inbred mice aged between 6-12 weeks old were used for all experiments. Marrow cells were harvested from CD1 mice by flushing freshly extracted femurs with HBSS (w/o Ca/Mg). Marrow flush was washed with PBS, filtered through a 40 micron strainer (BD) and triturated through a 21G needle prior to application on a histopaque gradient. Neutrophils were isolated after red cell lysis (0.2 % NaCl for 45 seconds followed by 1.6% NaCl) of the cell pellet. Monocytes were isolated with a CD11b positive magnetic sort (Miltenyi Biotec CD11b microbeads) of the mononuclear cell layer after washing in ice cold PBS with 0.02mM EDTA.

Primary murine immune cells were cultured for 6 days in L929 media. Labelling with compounds **5** and **6** was carried out at room temperature for 10 min, washed three times, and then incubated for 24 h. Cell viability was determined using two methods:

- MTT assay: followed standard procedures (see results in Fig S2).
- TO-PRO-3 assay: after the incubation time, cells were incubated with cell-impermeable DNA-binding dye TO-PRO-3 and then analysed by flow cytometry. Quantified cell death by To-Pro 3 incorporation showed no increase in cell death in labelled versus unlabelled cells (Fig S3).

2.2. Flow cytometry analysis

Detached HeLa cells were seeded in a 24-well plate at a density of 3.5×10^4 cells/well (350 μ L per well). Cells were incubated (37°C / 5% CO₂) for 24 h prior to peptoid treatment at concentration of 5 μ M for 15 and 30 min. After desired incubation time, media were removed and cells washed with PBS. HeLa were detached by trypsination at 37°C (trypsin/EDTA, 80 μ L per well) and collected in 2 % FBS/ PBS with or without 0.2 % of trypan blue (final volume per well of 300 μ L). Samples were analysed by flow cytometry using the 633 nm JDS

Uniphase™ HeNe air-cooled (750-810 nm for Cy 7 and for Cy5.5) the BD Biosciences FACSDiva software.

2.3. Confocal imaging of cells

Labelled cells (2×10^6) were seeded onto coverslips (no. 1.5, borosilicate glass) and allowed to adhere before being fixed in a formaldehyde solution [from 4% paraformaldehyde (weight/vol)] in phosphate-buffered saline (PBS) [NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 1.8 mM, 5 mM MgCl₂, 10 mM EGTA, 4% Sucrose (weight/vol), pH 7.4]. Cells were permeabilized by the addition of 0.006% Saponin (4°C; 30 minutes), washed twice in 50 mM NH₄Cl (in PBS), and blocked (10% fetal bovine serum, 0.5% bovine serum albumin in PBS) for 30 min. Subsequent washes and staining were performed in PBS containing 10% fetal bovine serum and 0.5% bovine serum albumin. Cell nuclei were stained using DAPI (10ug/ml; 10 minutes). Coverslips were mounted using Prolong Gold (Molecular probes).

A laser-scanning confocal imaging system (TCS SP5; Leica), incorporating a DMI 6000 inverted microscope (63× oil-immersion objective) was used for image acquisition. ImageJ was used for image processing. Cy5.5 was excited with a dedicated 633 nm line, and emitted light filtered using spectral separation slits (680-770nm), whereas DAPI was excited with a dedicated 405 nm UV diode, and emitted light filtered using spectral separation slits (415-490nm). Images were obtained by Kalman averaging of at least two individual scans, and images were obtained sequentially and merged off-line.

3. *In vivo* assays

3.1. Adoptive transfer experiments

All animal procedures were performed with ethical review and licences. CD1 inbred mice aged between 6-12 weeks old were used for all experiments. Retrieved cells were labelled with the corresponding dye-labelled peptoids at room temperature for 10 min prior to three washes. The washes were checked for residual fluorescence using appropriate filters on the Xenogen Spectrum. 3 million cells were injected through the lateral tail vein of mice in buffer of PBS with 10% BSA.

3.2. Optical whole body murine imaging

All animal procedures were performed with ethical review and licences. CD1 inbred mice aged between 6-12 weeks old were used for all experiments. *In vivo* optical imaging was performed on the Xenogen Spectrum (Caliper LS) and the Visen FMT 2500. Filters and imaging channels were aligned with Cy5.5 and Cy7. Imaging exposure for reflectance fluorescence (Xenogen) was for 5 seconds, large binning, f stop 2, filters at 680/720 for Cy5.5 and 745/820 for Cy7. For FMT 2500, channels 680nm and 750 were used for Cy.5.5 and Cy7 respectively. Animals received general anaesthesia with intraperitoneal ketamine and metomidate. For luminescent imaging on the Xenogen Spectrum, a block was placed in the excitation filter and emission filter kept open for 5 min at large binning.

3.3 . Subcutaneous inflammation model

50 μ g of *E.coli* LPS O55:B5 (Sigma) was mixed with ice cold matrigel (BD) and injected subcutaneously as previously described⁴. Contralateral flanks were injected with LPS matrigel implants. For luminol derived imaging, sodium salt luminol (200mg/kg, Sigma) was injected intraperitoneally and imaging commenced 15 minutes later.

Reference:

4- Gross, S., et al. (2009) *Nat. Med.* 15, 455-461.

4 Supplementary Results

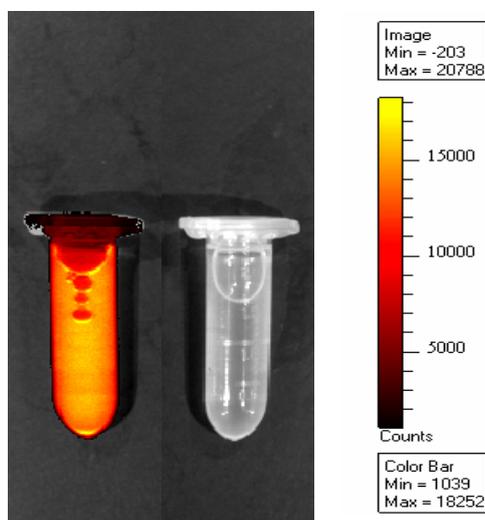


Figure S1. Fluorescent imaging of an Eppendorf vial containing the Cy5.5-9mer labelled neutrophils (concentration: 10^6 cells/mL) and an Eppendorf vial of control buffer (excitation 680 nm; emission 740 nm).

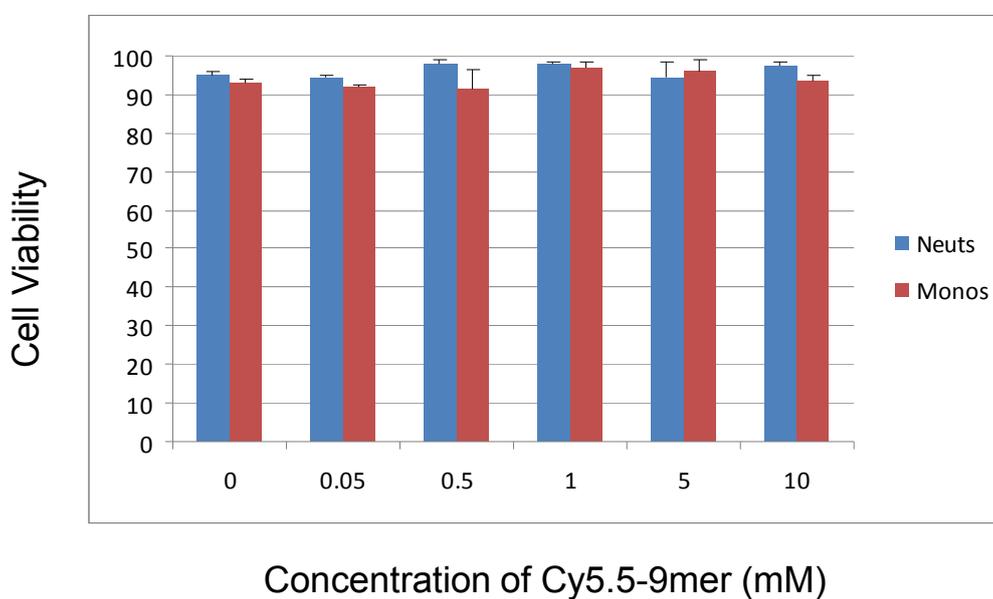


Figure S2. Cell viability (MTT) assay of neutrophils and monocytes after labelling with Cy5.5-9-mer at different concentrations.

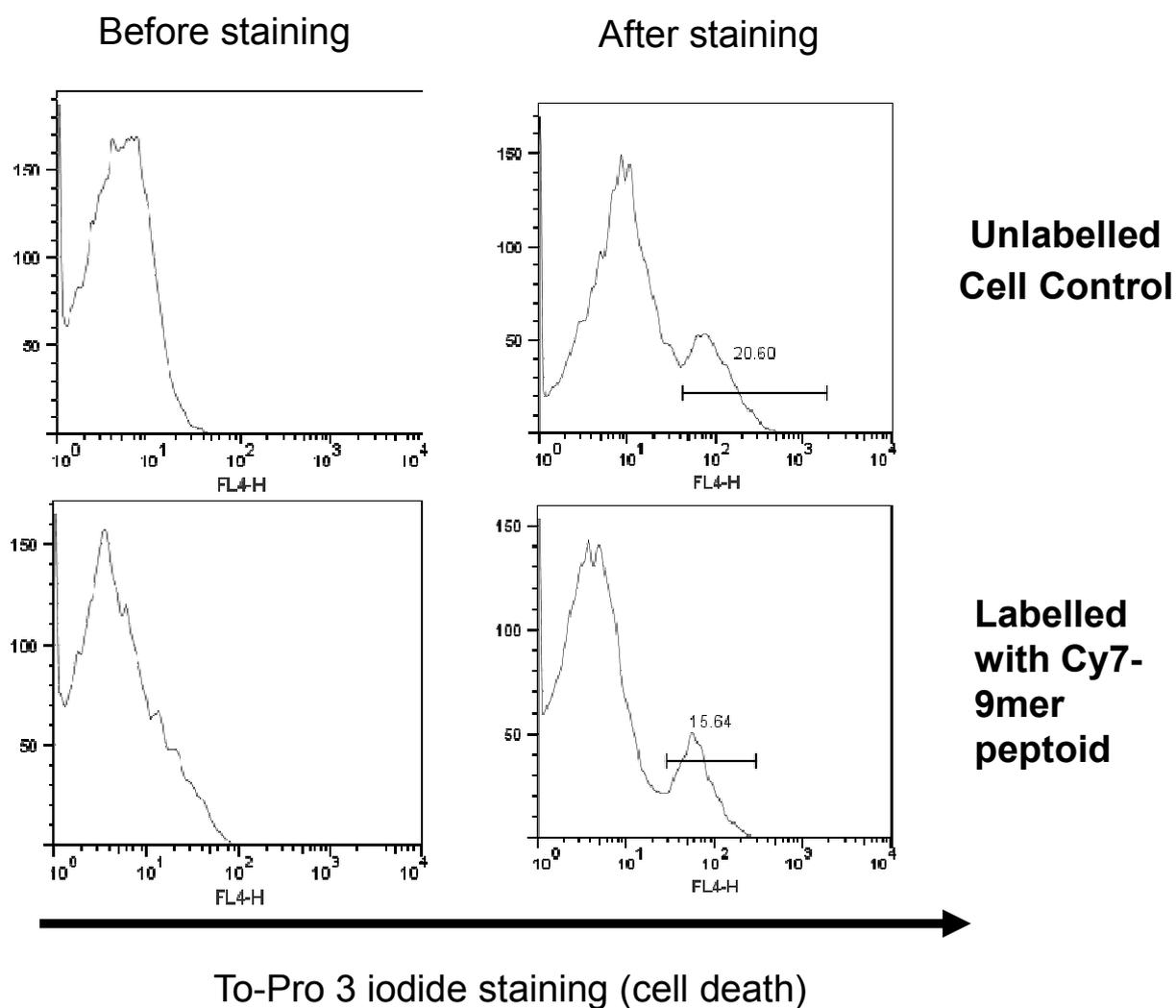


Figure S3. Flow cytometry analysis of Cy7-9mer labelled neutrophils after incubation with TO-PRO-3.