Supporting Information

Site-selective installation of BASHY fluorescent dyes to Annexin V for targeted detection of apoptotic cells

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1. General Remarks

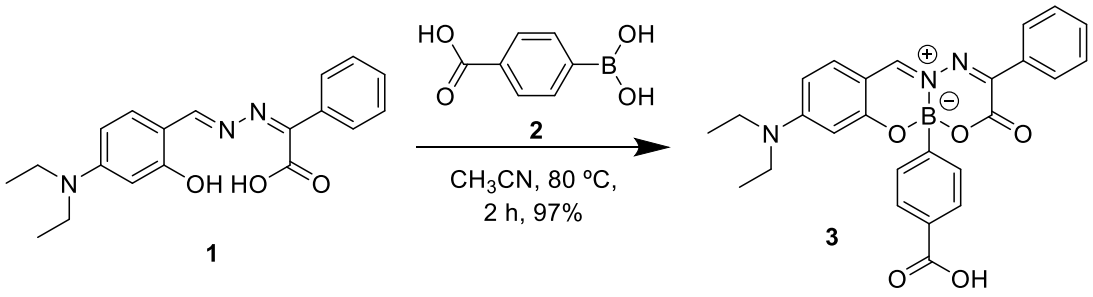
Acetonitrile was used without any purification, dimethylformamide (DMF) was distilled under reduced pressure and dried over molecular sieves and trimethylamine was dried with calcium hydride under nitrogen atmosphere and freshly used. 4-carboxyphenylboronic acid (**2**) and O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TBTU) were purchased from Sigma-Aldrich. Ligand **1** was prepared according to reference **6** of the manuscript and 2-azidoethanamine was synthesized according to the reference *Biomacromolecules* **2013**, *14*, 4407–4419.

NMR spectra were recorded in a Bruker Fourier 300 using $CDCl_3$, $(CD_3)_2SO$ as deuterated solvents and $(CH_3)_4Si$ (^1H) as internal standard. All coupling constants (*J* values) are expressed in Hz and chemical shifts (δ) in ppm. Multiplicities are given as: s (singlet), d (doublet), dd (double doublet), dt (double triplet), t (triplet), td (triple triplet), tt (triple triplet), q (quartet), quint (quintuplet) and m (multiplet). Mass spectra were recorded in a mass spectrometer (Micromass Quattro Micro API, Waters, Ireland) with a Triple Quadrupole (TQ) and with an electrospray ion source (ESI) operating in positive mode. Elemental analysis was performed in a Flash 2000 CHNS-O analyzer (ThermoScientific, UK).

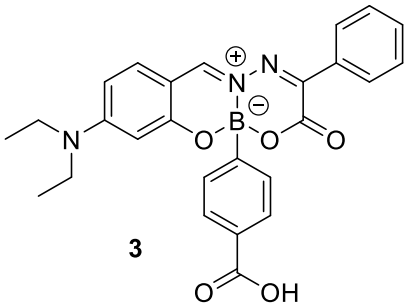
Reagents and solvents used for Annexin V conjugations were obtained from Sigma-Aldrich and used without any prior purification. Sulfo-cyanine5 azide (Cy5-N_3) was bought from Lumiprobe and used without any prior purification. Mass spectra were acquired on a high-mass Q-TOF-type instrument Xevo G2-S (Waters, Manchester, UK). Mass spectrometry experiments were performed at a capillary voltage of 1990 V, cone voltage of 80 V and source offset voltage of 80 V. Spectra were processed using MassLynx V4.1 (Waters).

2. Chemical Synthesis of BASHY Dyes

2.1 Synthesis of BASHY dye 3

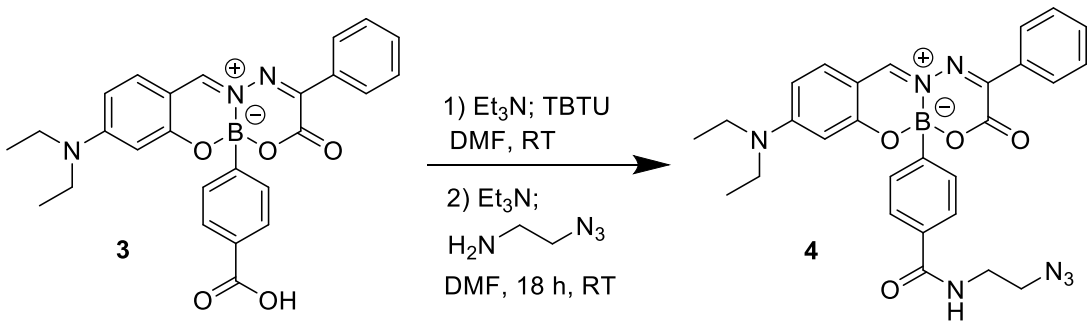


To assemble BASHY dye **3**, equimolar amounts of the ligand and 4-carboxyphenylboronic acid (0.1 mmol) were added to a round bottom flask, dissolved in 1 mL of acetonitrile and stirred at 80 ºC for 2 h. Then, the volatiles were evaporated and the obtained solid was washed with 0.5 mL of cold methanol in order to remove minor impurities.

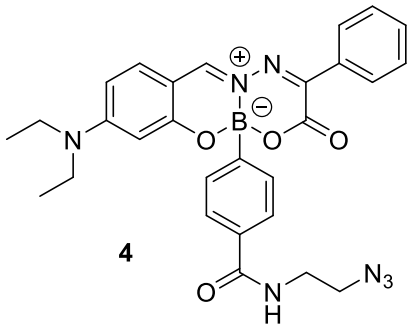


Compound **3** is an orange solid and was isolated with 97% yield:
^{1}H NMR (300 MHz, DMSO) δ 12.76 (s, 1H, -COOH), 8.93 (s, 1H, -Ph(C<u>H</u>)=N-), 7.88 – 7.81 (m, 2H, Ar), 7.78 (d, *J* = 8.1 Hz, 2H, Ar), 7.56 – 7.40 (m, 4H, Ar), 7.35 (d, *J* = 8.4 Hz, 2H, Ar), 6.61 (dd, *J* = 9.0, 2.1 Hz, 1H, Ar), 6.25 (d, *J* = 2.1 Hz, 1H, Ar), 3.64 – 3.41 (m, 4H, -N(C<u>H$_2$</u>)$_2$(CH$_3$)$_2$), 1.13 (t, *J* = 6.9 Hz, 6H, -N(CH$_2$)$_2$(C<u>H$_3$</u>)$_2$).
^{13}C NMR (75 MHz, DMSO) δ 167.47, 160.51, 157.27, 155.14, 152.19, 135.67, 132.42, 130.81, 130.43, 130.05, 128.95, 128.54, 128.32, 108.34, 106.02, 97.53, 44.82, 12.62.
ESI: $[M+H]^{+}_{obs}$: 470; $[M+H]^{+}_{calc}$: 470.2.
Elemental analysis: calcd (%) for $C_{26}H_{24}BN_3O_5$: C 66.54, H 5.15, N 8.95, found (%): C 66.17, H 5.40, N 9.14.

2.2 Synthesis of BASHY dye 4



BASHY dye **3** (0.181 mmol) was dissolved in 2 mL of dry dimethylformamide (DMF) and triethylamine (0.217 mmol). The mixture was stirred at room temperature for 10 min and then TBTU (0.217 mmol) was added. After 30 min, triethylamine (0.235 mmol) and the 2-azidoethanamine (0.217 mmol) were added and the reaction mixture was left to react over 18 h at room temperature. DMF was evaporated and 20 mL of ethyl acetate was added into the crude mixture. Organic phase was washed with water (2 x 20 mL), dried with anhydrous sodium sulfate, filtered and the volatiles were evaporated under reduced pressure. The solid obtained was washed with cold MeOH (2 x 0.75 mL) affording BASHY dye **4** with high yield and purity.



Compound **4** is an orange solid and was isolated with 91% yield:
^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (s, 1H, -Ph(C<u>H</u>)=N-), 8.03 – 7.89 (m, 2H, Ar), 7.59 (d, *J* = 8.1 Hz, 2H, Ar), 7.48 – 7.32 (m, 5H, Ar), 7.23 (d, *J* = 9.3 Hz, 1H, Ar), 6.52 (t, *J* = 5.4 Hz, 1H, -NH-), 6.37 (dd, *J* = 9.3, 2.1 Hz, 1H, Ar), 6.17 (d, *J* = 2.1 Hz, 1H, Ar), 3.58 – 3.35 (m, 8H, 4xC<u>H</u>$_2$), 1.22 (t, *J* = 7.2 Hz, 6H, -N(CH$_2$)$_2$(C<u>H</u>$_3$)$_2$).
^{13}C NMR (75 MHz, $CDCl_3$) δ 168.31, 161.46, 157.56, 155.82, 153.89, 153.73, 134.82, 133.41, 132.58, 131.13, 131.07, 129.50, 128.35, 126.24, 108.09, 106.47, 98.72, 51.00, 45.54, 39.39, 12.83.
ESI: $[M+H]^+_{obs}$: 538; $[M+H]^+_{calc}$: 538.2.
Elemental analysis: calcd (%) for $C_{28}H_{28}BN_7O_4$: C 62.58, H 5.25, N 18.25, found (%): C 62.67, H 5.39, N 18.11.

3. Photophysical properties of BASHY dyes

The UV/vis absorption and corrected fluorescence spectra were obtained for aerated solutions at room temperature (absorbance *ca.* 0.1-0.2), using a UV-1603 spectrometer from Shimadzu and a Varian Cary Eclipse fluorimeter, respectively. Time-resolved fluorescence measurements (λ_{exc} = 442 nm) were done with a time-correlated single-photon-counting setup from Edinburgh Instruments (model FLS 920). The fluorescence quantum yields were measured relative to *N*-propyl-4-amino-1,8-naphthalimide (Φ_{fluo} = 0.48 in aerated acetonitrile, calibrated against quinine sulfate in 0.05 M sulfuric acid; Φ_{fluo} = 0.55).

3.1 BASHY dye 3

Table S1. Photophysical data of BASHY dye **3** in air-equilibrated solution.

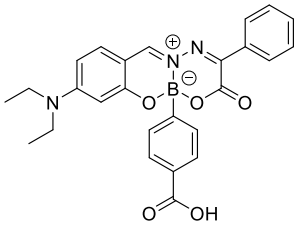
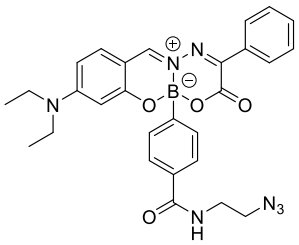
	solvent	$\lambda_{max,\ abs}$ (nm)	$\lambda_{max,\ fl}$ (nm)	Φ_{fl}	τ_{fl} (ns)	$\varepsilon_{abs,\ max}$ ($M^{-1}cm^{-1}$)
	CH_3CN	471	537	0.06	0.36	58600
	$CHCl_3$	477	516	0.58	2.92	61200

Figure S1. UV/vis absorption (solid lines) and fluorescence spectra (dashed lines) of BASHY dye **3** in acetonitrile (black) and in chloroform (red).

3.2 BASHY dye 4

Table S2. Photophysical data of BASHY dye **4** in air-equilibrated solution.

	solvent	$\lambda_{max,\ abs}$ (nm)	$\lambda_{max,\ fl}$ (nm)	Φ_{fl}	τ_{fl} (ns)	$\varepsilon_{abs,\ max}$ ($M^{-1}cm^{-1}$)
	CH_3CN	471	541	0.07	0.58	58500
	$CHCl_3$	478	519	0.60	3.39	56500

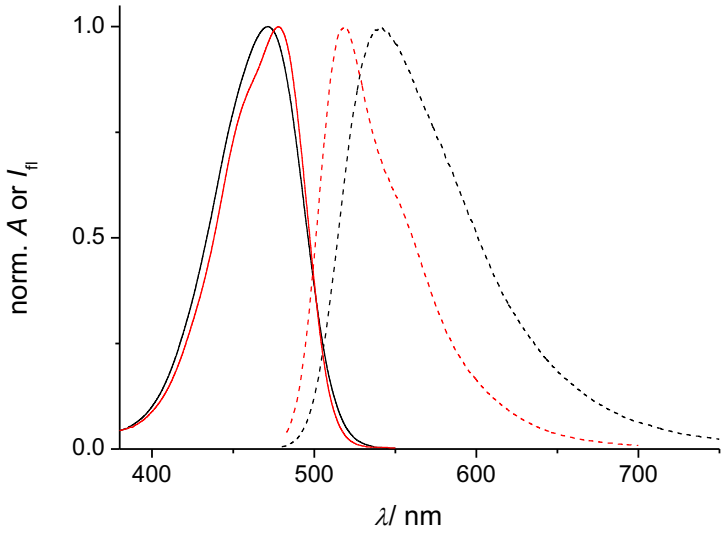


Figure S2. UV/vis absorption (solid lines) and fluorescence spectra (dashed lines) of BASHY dye **4** in acetonitrile (black) and in chloroform (red).

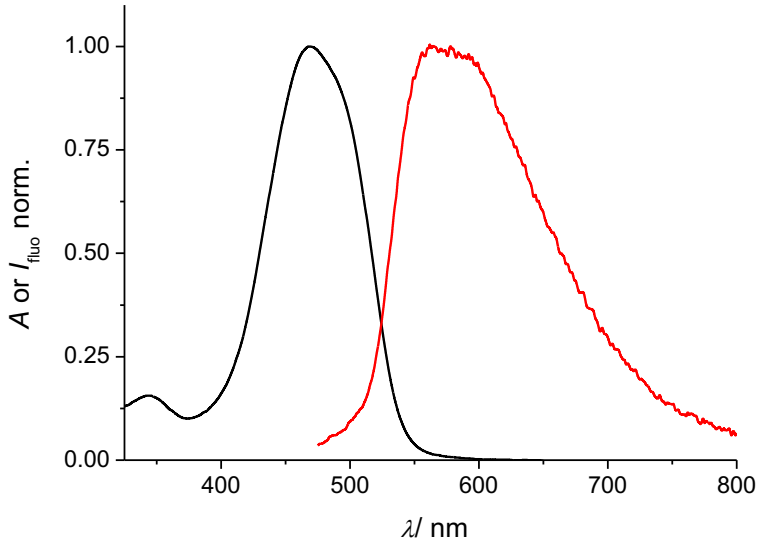


Figure S3. UV/vis absorption spectrum in black ($\lambda_{max,abs}$ = 470 nm) and corrected fluorescence emission spectrum in red ($\lambda_{max,fluo}$ = 575 nm, λ_{exc} = 470 nm) of BASHY dye **4** in 5% DMSO/NaPi (50 mM, pH 6). The fluorescence quantum yield was determined as Φ_{fluo} = 0.011.

3.3 BASHY dye 4 – reactivity with ethyleneglycol

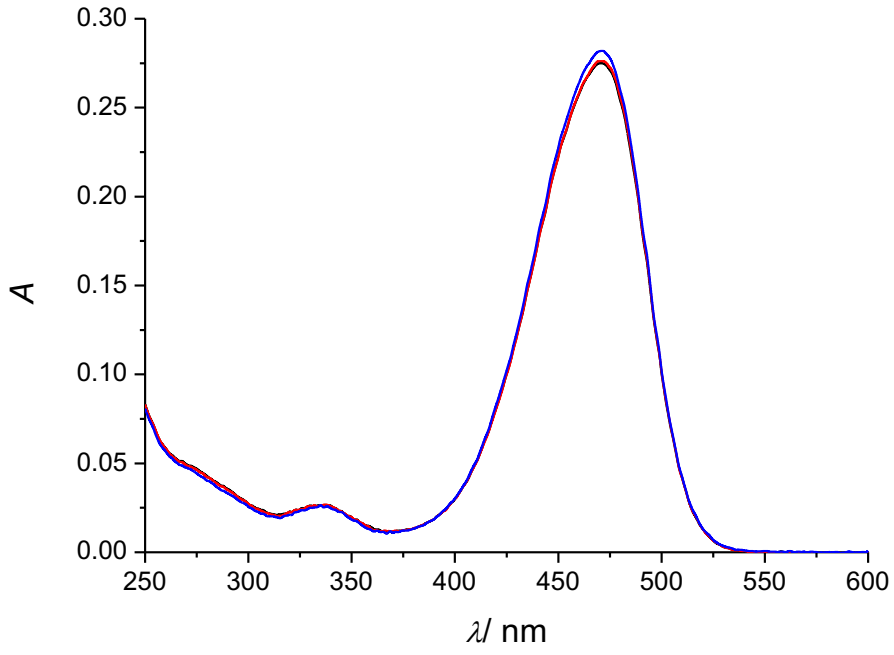


Figure S4. Stability assay of the probe in the presence of ethyleneglycol. The blue spectrum corresponds to BASHY dye **4** in absence of the 1,2-diol. The black spectrum was recorded immediately after addition of 5000 equivalents ethyleneglycol (t = 0 h) and the red spectrum was measured after an additional period of 24 h.

4. Cell viability assays of probe 4

4.1 Cell culture

Two different cell lines were used for the *in vitro* studies, namely Hek293T (a human embryonic kidney cell line) and HeLa cells (derived from cervical cancer cells). The cells were maintained in a humidified incubator at 37 ºC under 5% CO_2 and grown using 1x D-MEM (Dulbecco's modified Eagle medium) with Sodium Pyruvate and without L-Glutamine (Invitrogen, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Life Technologies), 1x MEM NEAA (Gibco, Life Technologies) ,1x GlutaMAX (Gibco, Life Technologies), 200 units/mL penicillin and 200 µg/mL streptomycin (Gibco, Life Technologies) and 10 mM HEPES (Gibco, Life Technologies).

4.2 Cell viability assay

10 000 cells/well were seeded in 96 well-plates and were treated with BASHY dye **4**, 24 h after the seeding, to allow the cells to stabilize. The cells were incubated with 1 µg/mL, 5 µg/mL and 10 µg/mL of the compound for 24 h. After this incubation period, the culture medium was removed and the cells were incubated with CellTiter-Blue (Promega) for 1 h 30 min at 37 ºC. The cells viability was evaluated by measuring the Emission Intensity in RFUs – relative fluorescent Units- with an Infinite M200 plate reader.

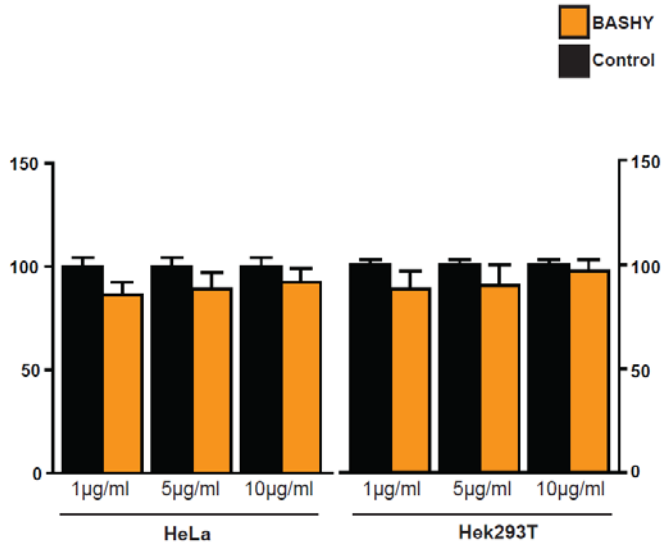


Figure S5. HeLa and Hek293T cells viability after treatment with 1 µg/mL, 5 µg/mL and 10 µg/mL of BASHY dye **4** for 24 h compared with the control treatment (medium + vehicle – DMF). The results correspond to 3 biological replicates (each with 3 technical replicates) and are shown as percentage of control (mean +sd).Differences were tested with a Mann-Whitney test that indicated no significant differences between cells treated with BASHY and the control.

5. Conjugation of DBCO-maleimide with Annexin V

5.1 Annexin V expression and purification

A colony of E. coli C41 (DE3) pLysS harboring the plasmid pET12a-PAPI_annexin was selected to inoculation of 100 mL LB medium containing ampicillin (100 µg/mL). The pre-culture was further incubated over night at 37 °C and 180 rpm. Aliquots of the overnight culture were stored in a 15% glycerol LB media at –20 °C. 20 mL of the overnight culture were used to inoculate 400 mL of LB medium containing ampicillin (100 µg/mL). Bacteria were grown to OD 0.6 (37 °C, 180 rpm) where upon expression was induced by 1 mM IPTG (Invitrogen). Expression of Annexin V was allowed for 4 h until cell culture was harvested by centrifugation at 4 °C and 7500 x g for 10 min. Supernatant was inactivated and discarded and the cells were resuspended in 50 mM Tris-HCl (pH 7.2) containing 1 mM *β*-mercaptoethanol and 10 mM $CaCl_2$. Cells were then lysed using a sonicator (Sonics Vibra-Cell, pulse on 30 s, pulse off 40 s, 2.5 min) and fragments were spinned down at 4 °C and 17000 x g for 45 min. Cell fragments were further resuspended in 50 mM Tris HCl (pH 7.2) containing 1 mM β-mercaptoethanol and 20 mM EDTA and spinned down once more at 4 °C and 17000x g for 20 min. The supernatant was collected and the protein was purified by FPLC (AKTA basic, GE Healthcare) using a size exclusion column (SepFast GF-HS M 16/60, Generon). The mobile phase was 20 mM Tris HCl (pH 8.0), flow rate 1 mL/min and fractions of 2 mL were collected and analysed by SDS-PAGE. A second round of purification was done with an anion exchange column (HighRes15 Q, Generon). The column was equilibrated with 20 mM Tris HCl (pH 8.0) at a flow rate of 1 mL/min. After loading the concentrated protein sample, the column was washed with 3 column volumes with the same buffer. A buffer gradient of 0–100% 1 M NaCl in 20 mM Tris HCl eluted the protein and fractions of 1 mL were collected. The fractions were analysed by SDS-PAGE and combined. Protein concentration was determined with a UV spectrometer at wavelength 280 nm.

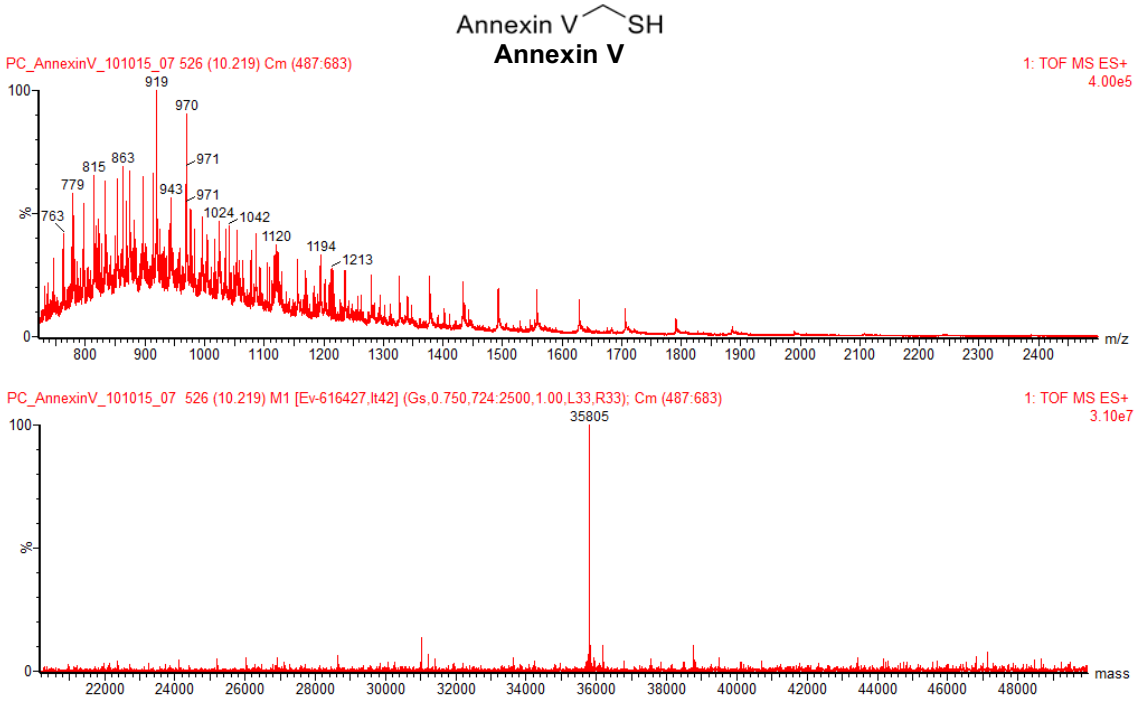
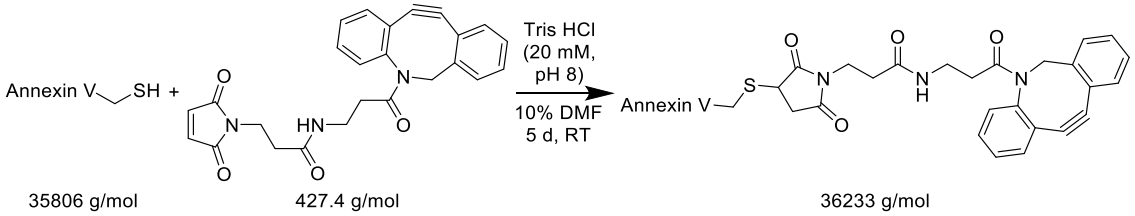


Figure S6. ESI–MS spectrum of Annexin V in TrisHCl (20 mM, pH 8).

5.2 Optimization of the bioconjugation reaction



In a 0.5 mL eppendorf, 8 µL of a 25 µM solution of Annexin V was added to a solution of 28 µL of TrisHCl (20 mM, pH 8) and 3.5 µL of DMF. After that, 0.5 µL of a solution of 10 mM DBCO-maleimide (in DMF) was added and the reaction was left stirring at room temperature for 5 days. After that, Zeba spin desalting column was used to purify the product, which was then concentrated using a vivaspin 500 (MWCO 5kDa). **Annexin V-mal-DBCO** was confirmed by LC-MS. Optimization of the reaction was made according to the conditions depicted in Table **S3** and the MS obtained depicted in Figure **S6**-**Figure S7**.

Table S3. Conditions used to modify 5 µM Annexin V in Tris HCl with DBCO-maleimide @ RT

Attempt	pH	Equivalents of Maleimide	% of DMF	Time of Evaluation (h)
1	7.0	25	1.0	6, 24 & 50
2	7.0	200	4.0	6 & 28
3	8.0	10	5.0	6 & 28
4	8.0	25	1.0	6 & 11
5	8.0	50	2.5	6 & 11
6	6.0	100	5.0	7, 24 & 32
7	6.0	200	10.0	7, 24 & 32
8	8.0	25	10.0	12 & 34
9	7.0	100	5.0	6 & 11
10	8.0	25	10.0	100 & 120

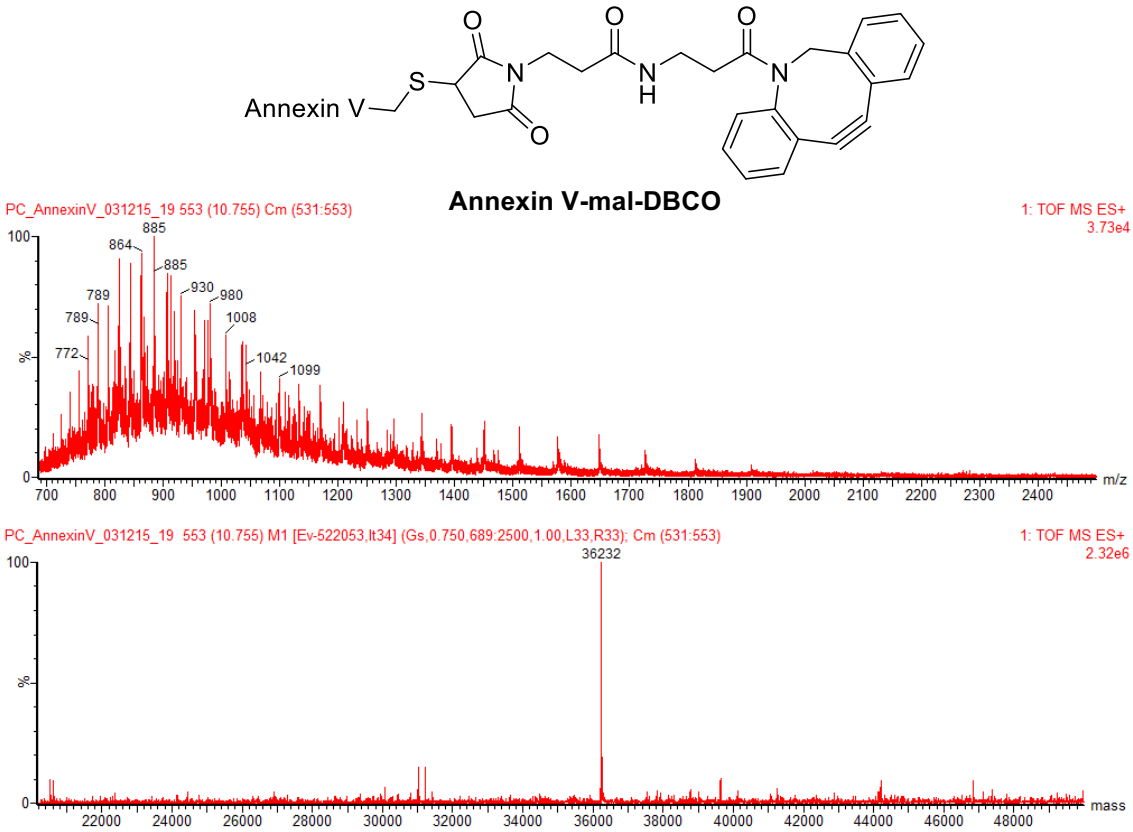


Figure S7. ESI–MS spectrum of the product from the reaction of Annexin V in TrisHCl (20 mM, pH 8) with 25 equivalents of DBCO-mal for 5 d at room temperature.

5.3 MS/MS data

10 µM solution of the purified **Annexin V-mal-DBCO** sample was digested overnight with trypsin. No reduction/alkylation step was performed.

All LC-MS/MS experiments were performed using a nanoAcquity UPLC (Waters Corp., Milford, MA) system and an LTQ Orbitrap Velos hybrid ion trap mass spectrometer (Thermo Scientific, Waltham, MA). Separation of peptides was performed by reverse-phase chromatography using a Waters reverse-phase nano column (BEH C18, 75 µm i.d. x 250 mm, 1.7 µm particle size) at flow rate of 300 nL/min. Peptides were initially loaded onto a pre-column (Waters UPLC Trap Symmetry C18, 180 µm i.d x 20mm, 5 µm particle size) from the nanoAcquity sample manager with 0.1% formic acid for 3 minutes at a flow rate of 10 µL/min. After this period, the column valve was switched to allow the elution of peptides from the pre-column onto the analytical column. Solvent A was water + 0.1% formic acid and solvent B was acetonitrile + 0.1% formic acid. The linear gradient employed was 5-40% B in 60 minutes.

The LC eluant was sprayed into the mass spectrometer by means of a New Objective nanospray source. All *m/z* values of eluting ions were measured in the Orbitrap Velos mass analyzer, set at a resolution of 30000. Data dependent scans (Top 20) were employed to automatically isolate and generate fragment ions by collision-induced dissociation in the linear ion trap, resulting in the generation of MS/MS spectra. Ions with charge states of 2+ and above were selected for fragmentation. Post-run, the data was processed using Protein Discoverer (version 1.4., ThermoFisher). Briefly, all MS/MS data were converted to mgf files and these were submitted to the Mascot search algorithm (Matrix Science, London UK) and searched against a custom database containing the annexin V protein sequence and common contaminant sequences and applying variable modifications of oxidation (M), deamidation (NQ) and a custom modification of dibenzocyclooctyne-maleimide (mal-DBCO, **5**), using a peptide tolerance of 25 ppm (MS) and 0.8Da (MS/MS). Peptide identifications were accepted if they could be established at greater than 95.0% probability. Significant hits which suggested that the mal-DBCO was bound to peptides were then verified by manual inspection of the MS/MS data. Mascot search showed that ALLLLCGEDD was detected with the expected modification on cysteine. The MS/MS spectrum is depicted in the following figure with the majority of the sequence ions assigned (Figure **S8**).

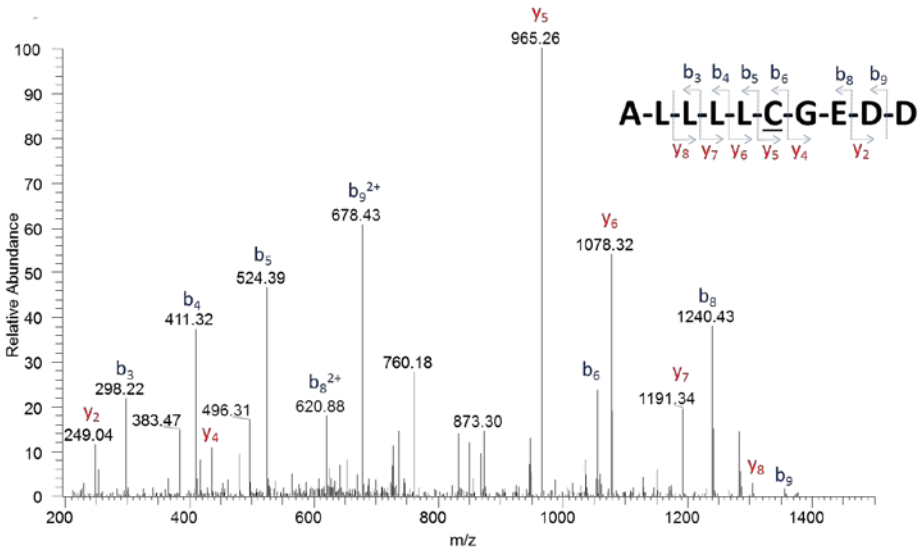
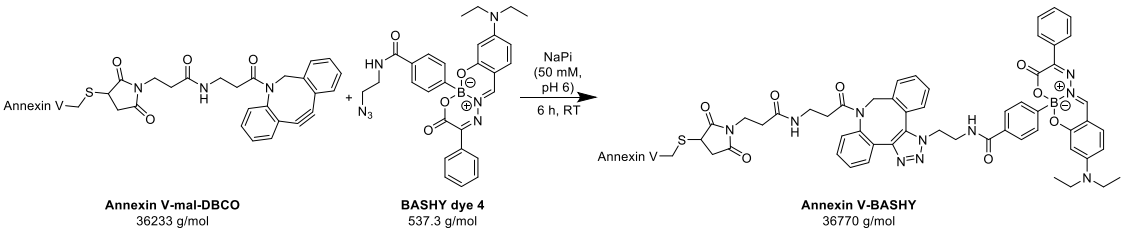


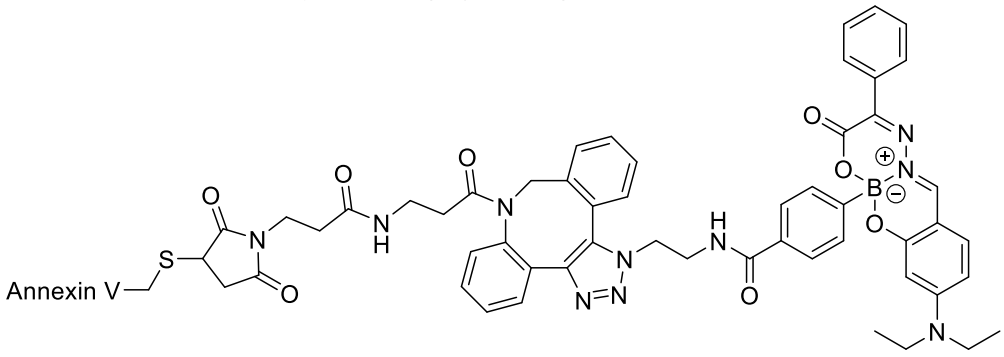
Figure S8. MS spectrum of the peptide ALLLLCGEDD obtained after tryptic digestion of the purified product and containing the modification at cysteine 315. *m/z* value of the doubly charged peptide = 744.84.

6. Click Chemistry on Protein Surface

6.1 Preparation of Annexin V-BASHY



In a 0.5 mL eppendorf, 20 µL of a 5 µM solution of Annexin V-maleimide-DBCO in NaPi (50 mM, pH 6) were mixed with 1.0 µL of a solution of 525 µM azide-BASHY (dye **4** in DMF) and the reaction was left stirring at room temperature for 6 h. After that, a Zeba™ spin desalting column was used to purify **Annexin V-BASHY** into NaPi (50 mM, pH 6), which was evaluated by LC-MS (Figure **S9**).



Annexin V-BASHY

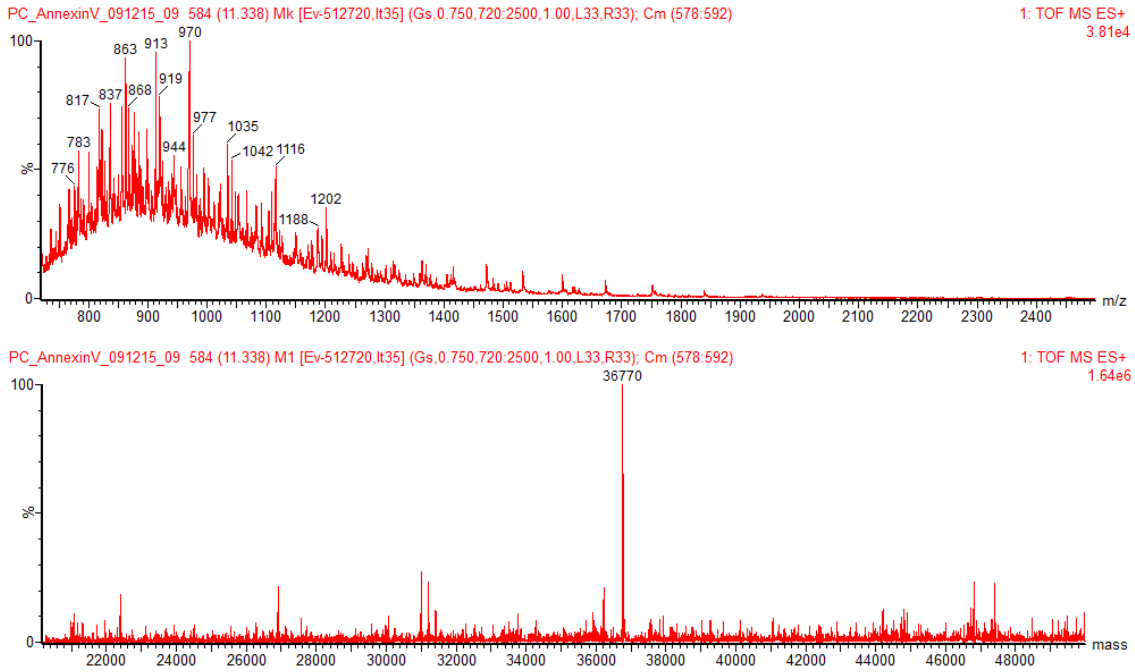


Figure S9. ESI–MS spectrum of the product from the reaction of Annexin V-mal-DBCO in NaPi (50 mM, pH 6) with 25 equivalents of BASHY dye **4** for 6 h at room temperature.

6.1.1 Structural analysis

Literature basis: J.B. Bertoldo *et al. Biochim Biophys Acta* **2011**, *1814*, 1120–1126.

Annexin V, Annexin V-mal-DBCO and Annexin V-BASHY were concentrated to 0.5 µM in buffer (20 mM NaPi, pH 7.4) and assessed by circular dichroism (CD). The CD measurements were recorded using a Chirascan Spectropolarimeter (Applied Photophysics) equipped with a Quantum TC125 temperature control unit (25 °C). The data was acquired in a 0.1 cm pathlength with a response time of 1 s, a per-point acquisition delay of 5 ms and a pre and post-scan delay of 50 ms. Spectra were averaged over three scans in a wavelength range from 190 nm to 260 nm and deconvoluted according to the equation.

$$[\theta] = (\theta_{[222]} \times 100 \times M) / (C \times l \times n)$$

where θ is the ellipticity in degrees, l is the optical path length in cm, C is the concentration of sample in mg/ml, M is the molecular mass and n is the number of residues in the protein.

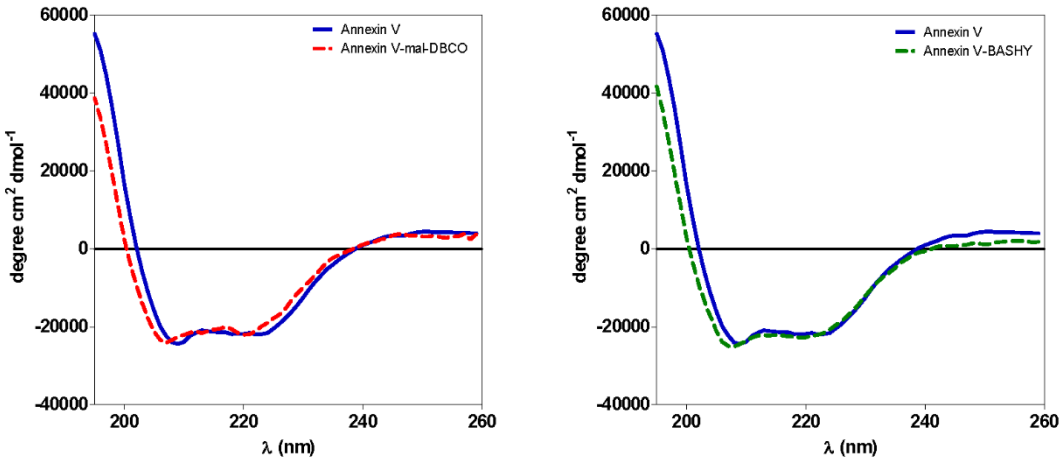
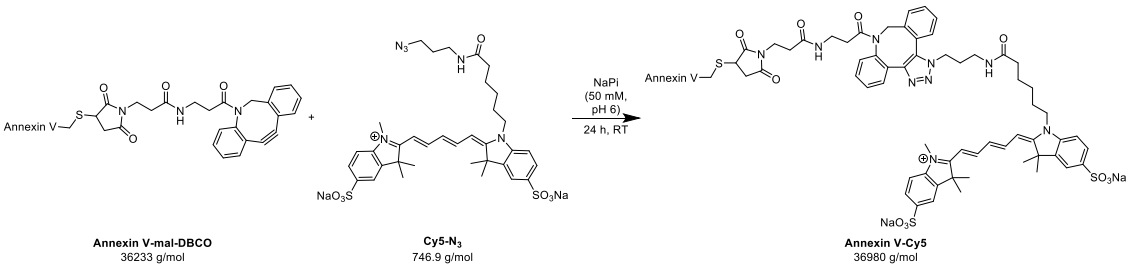


Figure S10. Circular dichroism (CD) spectra of Annexin V, Annexin V-mal-DBCO and Annexin V-BASHY (0.5 µM) in buffer (20 mM NaPi, pH 7.4).

6.2 Preparation of Annexin V-Cy5



In a 0.5 mL eppendorf, 25 µL of a 2.5 µM solution of purified Annexin V-maleimide-DBCO in NaPi (50 mM, pH 6) were mixed with 0.5 µL of a solution of 13.4 mM azide-Cy5 (in DMF). The reaction was left stirring at room temperature for 24 h. After that, a Zeba™ spin desalting column was used to purify **Annexin V-Cy5** into NaPi (50 mM, pH 6). The obtained product was evaluated by SDS-Page (Figure **S11**, lane II).

Gel Electrophoresis protocol:

Proteins were mixed with SDS sample buffer with 200 mM DTT (reducing buffer), heated for five minutes to 95 °C and separated on a 12% Tris glycine buffered SDS PAGE gel. After electrophoresis, BASHY labelled proteins were detected using the ChemiDoc XRS+ system with the 520DF30 emission filter, followed by standard Coomassie G250 staining. Results are shown in Figure **S11**.

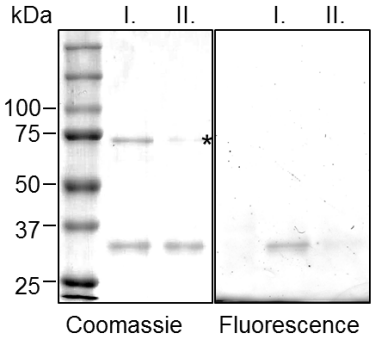
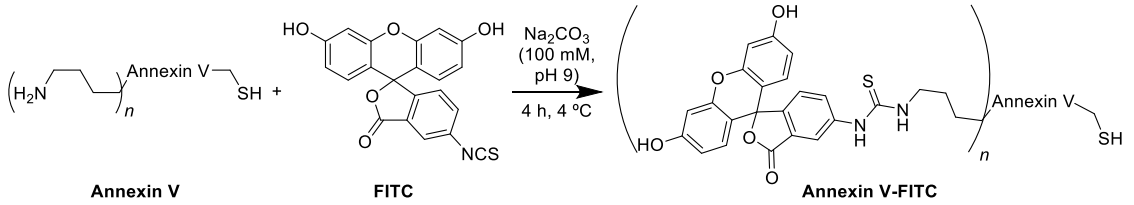


Figure S11. BASHY and Cy5 labelled AnnexinV was separated by SDS PAGE in reducing conditions (lanes I. and II.). Loading: 350 ng. BASHY fluorescence was detected prior to Coomassie staining using a ChemiDoc XRS+ system. * dimers

7. Preparation of Annexin V-FITC



Following a described protocol to label annexin V (wild type) with FITC and subsequent imaging of early and late apoptotic cells (*Nat Protoc.* **2009**; *4(9)*, 1383-95. doi: 10.1038/nprot.2009.143), a purified sample of **Annexin V-FITC** was isolated. Taking into account that this heterogenous lysine modification of Annexin V is already reported, only a gel electrophoresis was performed to characterize this construct.

Gel Electrophoresis protocol:

Proteins were mixed with SDS sample buffer with 200 mM DTT (reducing buffer) or without DTT (non-reducing buffer), heated for five minutes to 95 °C and separated on a 12% Tris glycine buffered SDS PAGE gel. After electrophoresis, FITC labelled proteins were detected using the ChemiDoc XRS+ system with the 520DF30 emission filter, followed by standard Coomassie G250 staining. Results are shown in Figure **S12**.

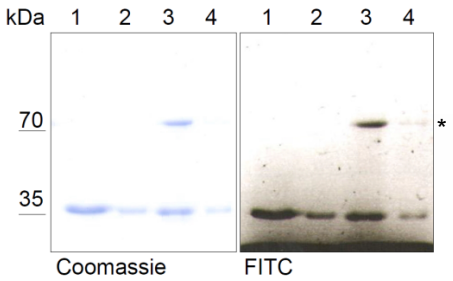


Figure S12. FITC labelled AnnexinV was separated by SDS PAGE in reducing (lanes 1 and 2) and non-reducing conditions (lanes 3 and 4). Loading: 1 µg (lanes 1 and 3), 200 ng (lanes 2 and 4). FITC fluorescence was detected prior to Coomassie staining using a ChemiDoc XRS+ system. * dimers

8. Cellular Imaging

Apoptosis assay protocol:

HeLa cells were grown to 80% confluency in DMEM supplemented with 10% FBS, GlutaMax (Gibco), MEM (Gibco), and 100 U/ml penicillin, 100 mg/ml streptomycin. To induce apoptosis 1 µM actinomycine D in DMSO was added to the medium while 0.1 % DMSO was added to the control. After 6 h, either 1 µg/mL of either Annexin V-BASHY, Annexin V-Cy5 or Annexin V-FITC was added for 15 min followed by 20 min of 4% paraformaldehyde fixation, 30 min of 100 µg/mL Hoechst 33342 staining and embedding in Fluoromount-G. Imaging was done using a Zeiss LSM 710 with identical laser, filter and detector settings for control vs. apoptosis samples. The blocking experiment followed exactly the same protocol, but using a presaturation of the receptors with a preincubation of non-fluorescent Annexin V-mal-DBCO (1 µg/mL) for 15 min. Results are shown in Figure **S13** to Figure **S19**. An evaluation of labelling efficiencies of both constructs bearing a single fluorophore was performed by calculating the ratio of labelled cells vs total cells (Figure **S20**).

8.1 Imaging with Annexin V-BASHY

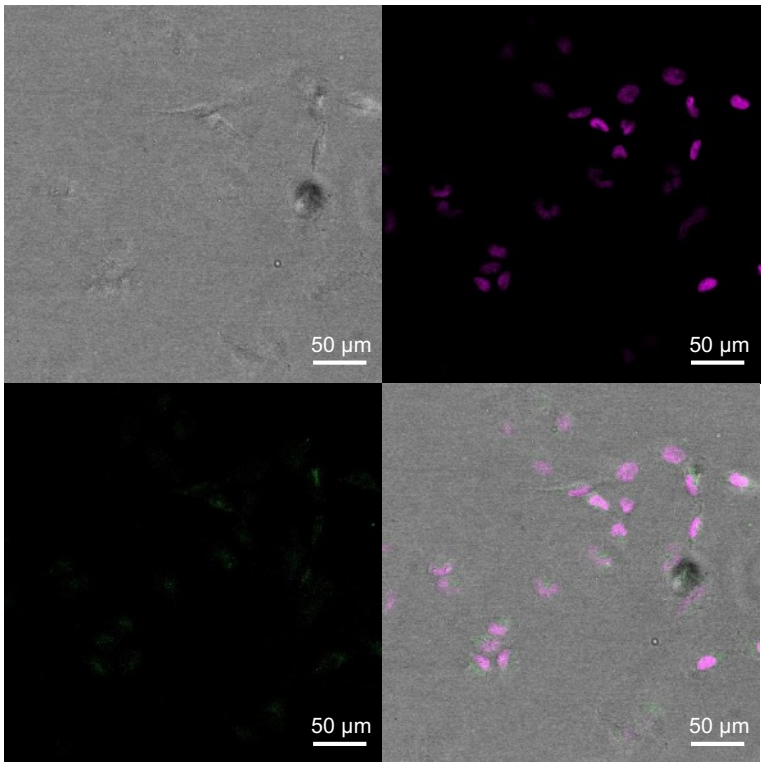


Figure S13. Confocal microscopy images of HeLa cells treated with the vehicle control (0.1% DMSO) followed by incubation with 1 µg/mL of Annexin V-BASHY for 15 min. Annexin V-BASHY (green), nuclear Hoechst staining (purple).

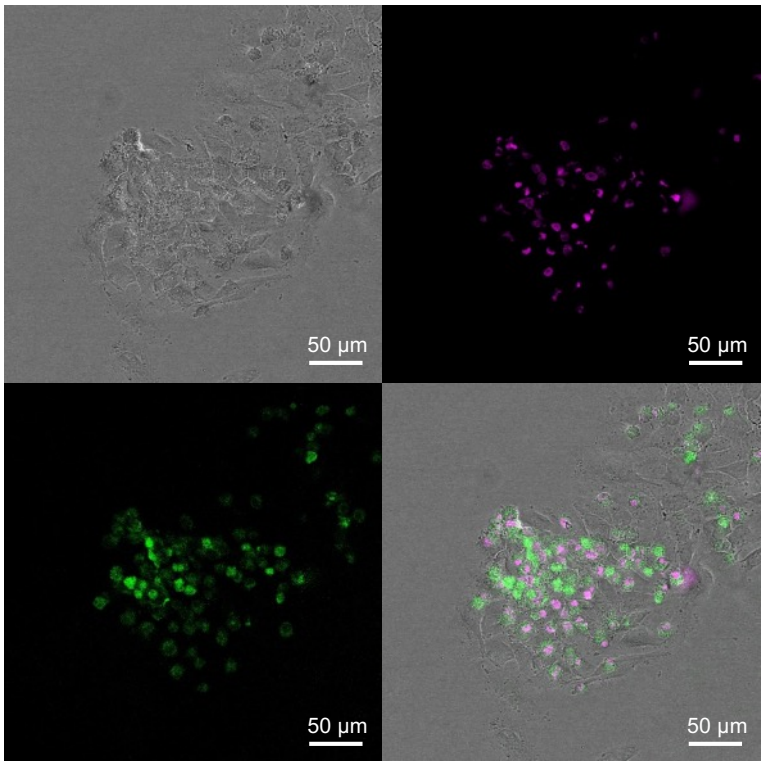


Figure S14. Confocal microscopy images of HeLa cells treated with 1 µM actinomycin D for 6 h to induce apoptosis followed by incubation with 1 µg/mL of Annexin V-BASHY for 15 min. Annexin V-BASHY (green), nuclear Hoechst staining (purple).

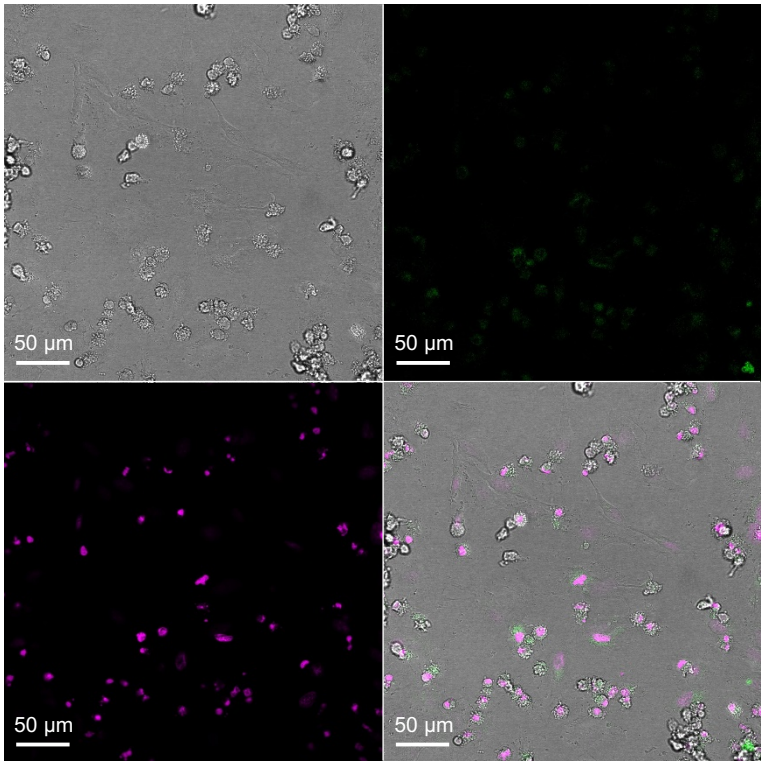


Figure S15. Blocking experiment. Confocal microscopy images of HeLa cells treated with 1 µM actinomycin D for 6 h to induce apoptosis followed by incubation with 1 µg/mL of Annexin V-mal-DBCO for 15 min and subsequent incubation with 1 µg/mL of Annexin V-BASHY. Annexin V-BASHY (green), nuclear Hoechst staining (purple).

8.2 Imaging with Annexin V-FITC

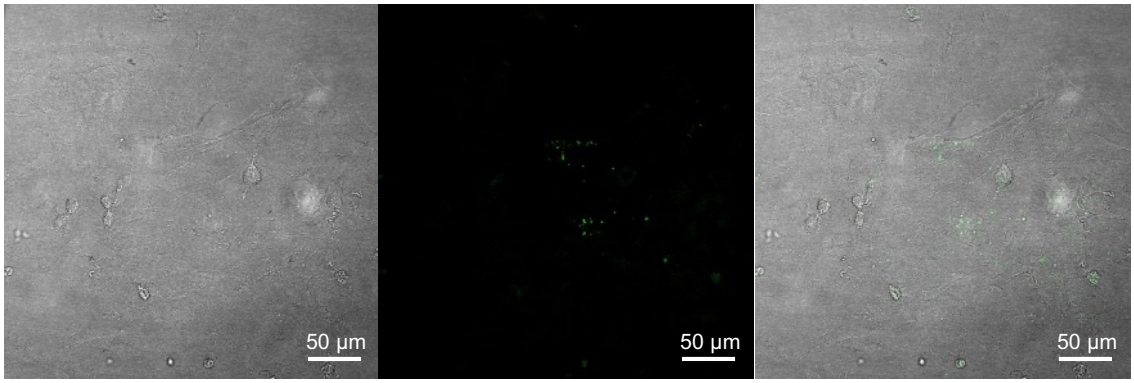


Figure S16. Confocal microscopy images of HeLa cells treated with the vehicle control (0.1% DMSO) followed by incubation with 1 µg/mL of Annexin V-FITC for 15 min. Annexin V-FITC (green).

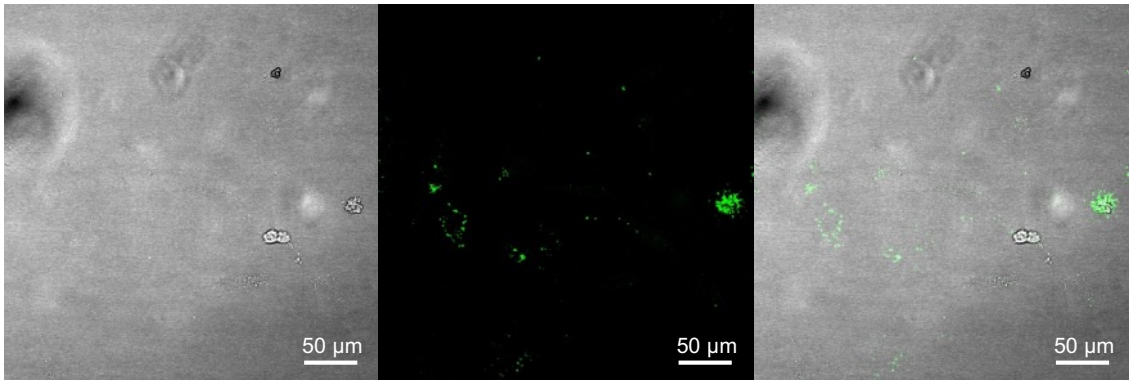


Figure S17. Confocal microscopy images of HeLa cells treated with 1 µM actinomycin D for 6 h to induce apoptosis followed by incubation with 1 µg/mL of Annexin V-FITC for 15 min. Annexin V-FITC (green).

8.3 Imaging with Annexin V-Cy5

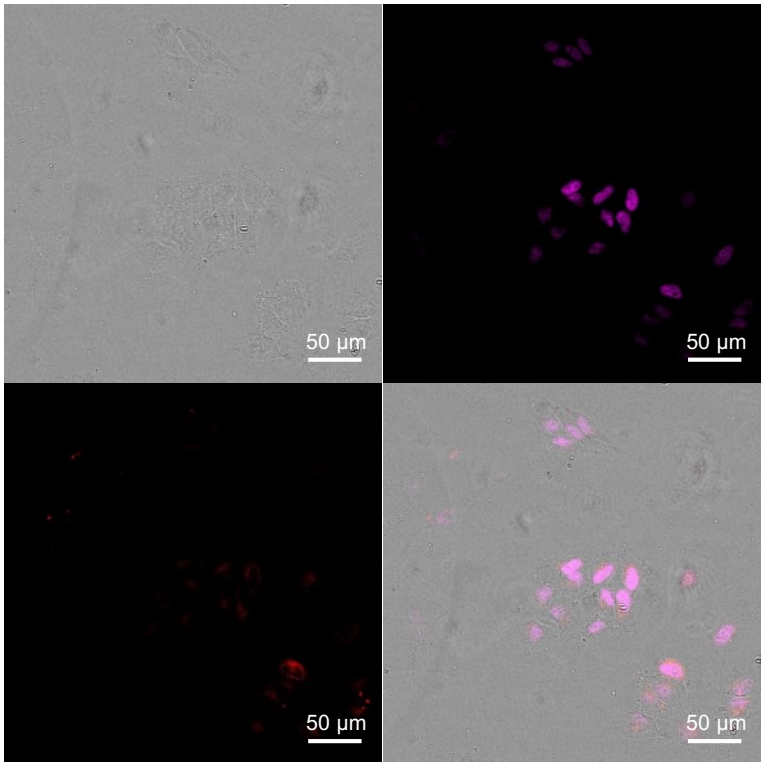


Figure S18. Confocal microscopy images of HeLa cells treated with the vehicle control (0.1% DMSO) followed by incubation with 1 µg/mL of Annexin V-Cy5 for 15 min. Annexin V-Cy5 (red), nuclear Hoechst staining (purple).

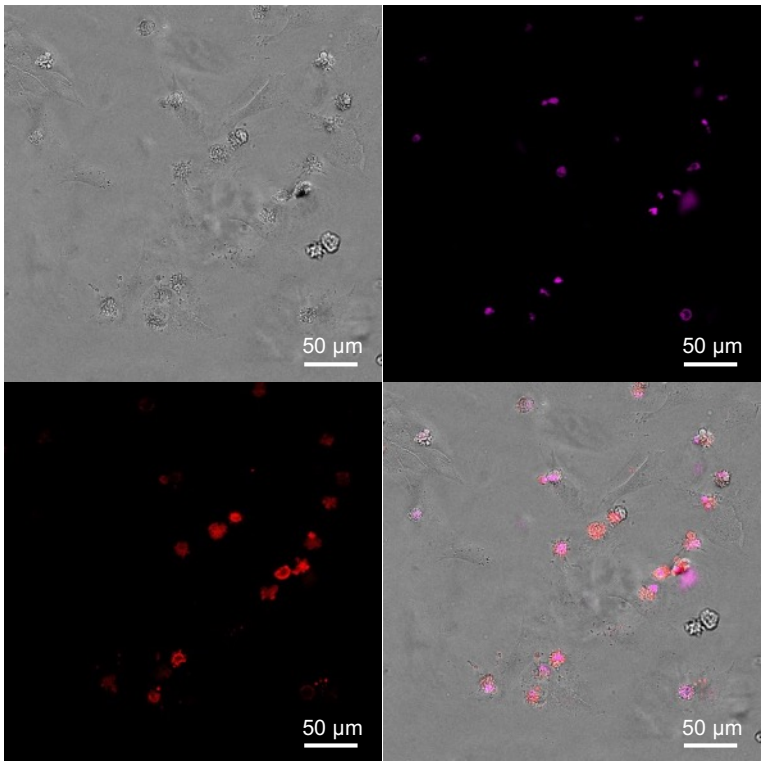


Figure S19. Confocal microscopy images of HeLa cells treated with 1 µM actinomycin D for 6 h to induce apoptosis followed by incubation with 1 µg/mL of Annexin V-Cy5 for 15 min. Annexin V-Cy5 (red), nuclear Hoechst staining (purple).

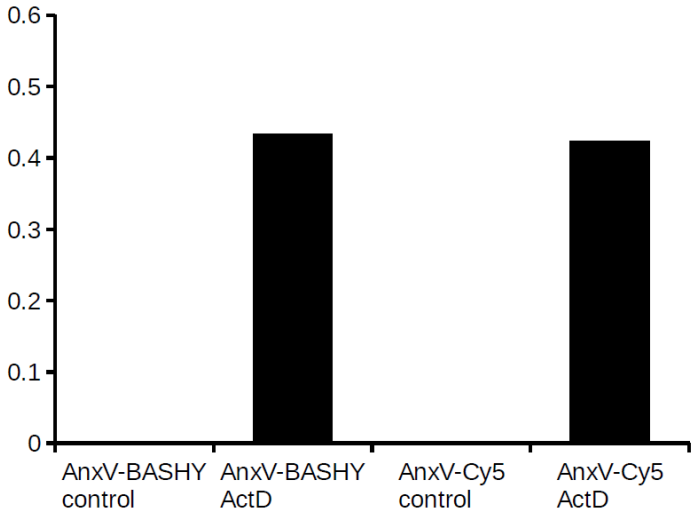


Figure S20. Labelling efficiencies for both site-selective Annexin V constructs. Ratio of labelled cells vs total cells of Annexin V-BASHY and Annexin V-Cy5 in the presence or absence of the apoptotic inducer (actinomycin D – ActD).