Supporting Information

A QUANTITATIVE METHOD TO DISTINGUISH CYTOSOLIC FROM ENDOSOME-TRAPPED CELL-PENETRATING PEPTIDES

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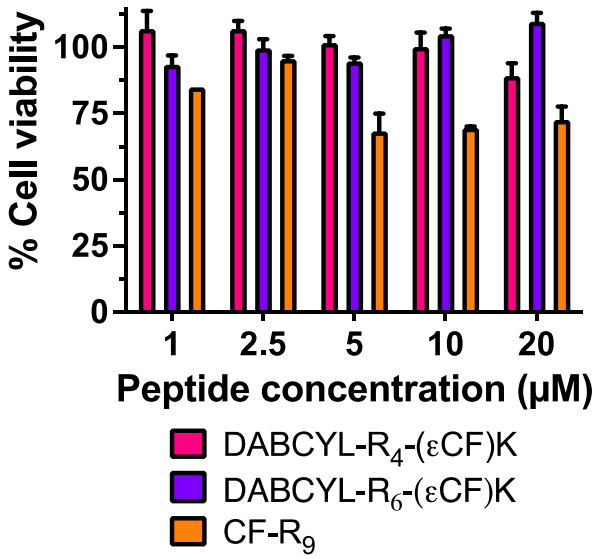
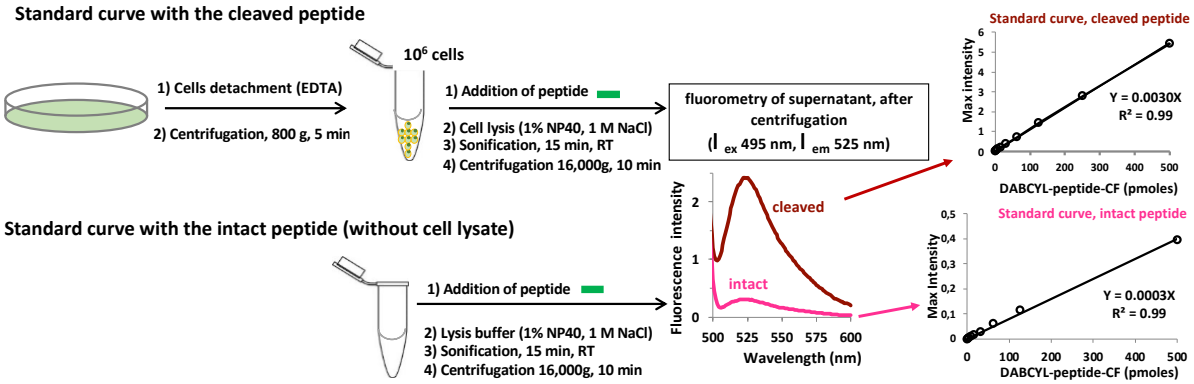


Figure S1. Cytotoxicity of the fluorescent peptides used in this study. Peptides were incubated for 1 hr with cells at the indicated concentrations. Experiments have been replicated 3 to 6 times independently.



Scheme S1. Fluorescence standard curves obtained with the cleaved peptide (with cell lysates) or the intact peptide. A range of 2-500 picomole peptide is incubated for 1 hr in lysis buffer (50 mM Tris pH7.4, 1% NP40, 1M NaCl) alone (control) or with cell lysates (eventually with addition of trypsin 0.008%), obtained with 10^6 cells in 200 µL lysis buffer, sonification for 15 min at RT and centrifugation at 16,000 g before fluorescence was recorded in the supernatant (λ_{ex} = 495 nm, λ_{em} = 525 nm). For every internalization assay, we ran in parallel this standard curve for the same peptide. See the Material and Methods section for detailed description.

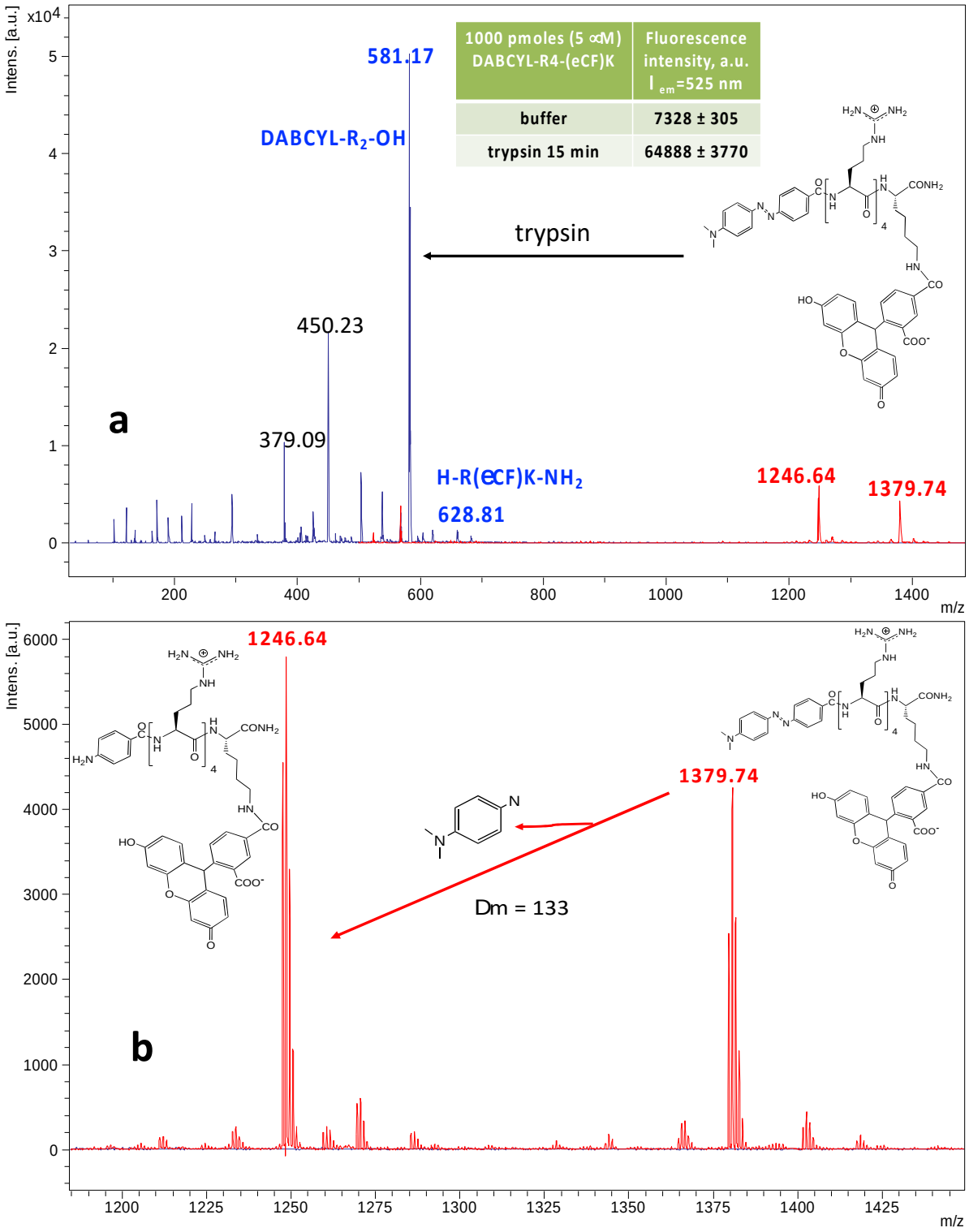


Figure S2. MALDI-ToF mass spectrometry and fluorescence intensity (λ_{em}=525 nm) of DABCYL-R4-(εCF)K treated or not with trypsin. (**a**) the MS spectrum in red corresponds to the intact peptide (1000 pmol, $[M+H]^+$ at m/z 1379.74 and 1246.64) in buffer; in blue the MS spectrum after incubation of the peptide (1000 pmol), with trypsin (0.0008%); the table shows the fluorescence signal obtained from the same samples; (**b**) in-source fragmentation observed on the DABCYL moiety, leading to the peptide loss of Δm = 133.

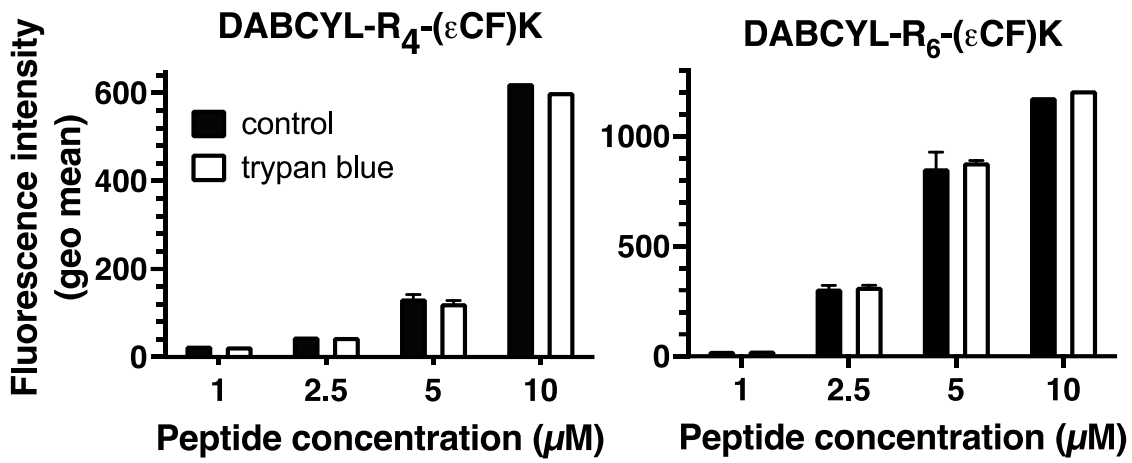


Figure S3. Flow cytometry analysis of DABCYL-R_n-(εCF)K internalization in the absence or presence of trypan blue. Experiments have been replicated 2 times.

Table S1. Ratio of internalization quantity between the oligoarginine peptides, measured either by flow cytometry or fluorometry.

	Internalization Ratio R_6 / R_4 Flow Cytom.	Internalization Ratio R_6 / R_4 Fluorometry	Internalization Ratio R_6 / R_9 Flow Cytom	Internalization Ratio R_6 / R_9 Fluorometry
1 ∝M, 37°C	1.7	3.5	0.47	1.2
1 ∝M, 4°C	1.7	1.5	1	1
2.5 ∝M, 37°C	9.5	12	1.2	1.2
2.5 ∝M, 4°C	3.5	2	1.1	1.2
5 ∝M, 37°C	7	7	1.4	1.7
5 ∝M, 4°C	3.9	3	1.1	1.5
10 ∝M, 37°C	1.7	5	1.4	2.9
10 ∝M, 4°C	5	4.5	1.8	1.8

Table S2. Protocol to determine quantitatively the peptide internalized by endocytosis, translocation and potentially endosomal escape: [a] Incubation at 37°C with peptides followed by lysis and degradation of all intracellular peptide, allows to measure the whole internalized peptide, either by endocytosis or translocation; [b] the same experiment followed by boiling the lysate to inhibit enzymes released and prevent intact peptide from degradation, gives access to the peptide internalized by endocytosis without the peptide that could escape endosomes; [c] subtracting [b] from [a] gives the quantity coming from translocation and endosomal escape; [d] running incubation at 4°C, followed by peptide enzymatic degradation (sonification + trypsin) allows to quantify translocation only with the cleaved peptide standard curve; [e] running incubation at 4°C, followed by boiling to keep peptide intact, allows to quantify only translocation with the uncleaved peptide standard curve. [f] Subtracting [d] or [e] from [c] gives the quantity of peptide that could escape endosomes.

Protocol	Phenomenon observed [g]
[a], 37°C incubation, sonification, trypsin	E + T
[b], 37°C incubation, boiling	E - EE
[c] = [a]-[b]	T + EE
[d], 4°C incubation, sonification, trypsin	T
[e], 4°C incubation, boiling	T
[f] = [c]-[d] or [c]-[e]	EE

[g] E, T and EE stand respectively for endocytosis, translocation and endosomal escape, processes that can be quantified according to kinetics of peptide incubation with cells.

Table S3. Quantity (pmoles) of DABCYL-R_2WR_2-(εCF)K internalized into cells, according to the experimental conditions described in the table of Scheme 1. Different amounts (pmoles) of peptides were incubated for 1 hr with 10^6 cells, either at 37°C (endocytosis and translocation, E+T) or 4°C (translocation, T). After washings, cells were submitted to lysis in conditions allowing complete peptide hydrolysis (columns a and d) or not (columns b and e). Column e corresponds to the quantification of the translocation process, calculated with the calibration curve of the intact peptide (see Scheme S1). Comparison, between columns permits to quantify only endocytosis (E) or translocation (T) contribution. The quantities of peptide incubated with cells at 200, 500, 1000 and 2000 pmoles correspond respectively to 1, 2.5, 5 and 10 μM extracellular concentrations. Measurements of intracellular pmoles of peptides correspond to μM intracellular concentrations.

R_2WR_2 pmoles [g]	(a) 37°C Lysate +trypsin RT (E+T)	(b) 37°C Boiled lysate (E)	(c) a-b 37°C (T)	(d) 4°C Lysate +trypsin RT (T)	(e) 4°C Lysate 4°C (T)	a-d (E)
200	3.1	0.8	2.4	2.0	-	1.1
500	13.6	4.1	9.5	6.8	-	6.8
1000	65	52	13	11.8	-	53,2
2000	223	67	156	20.3	-	203

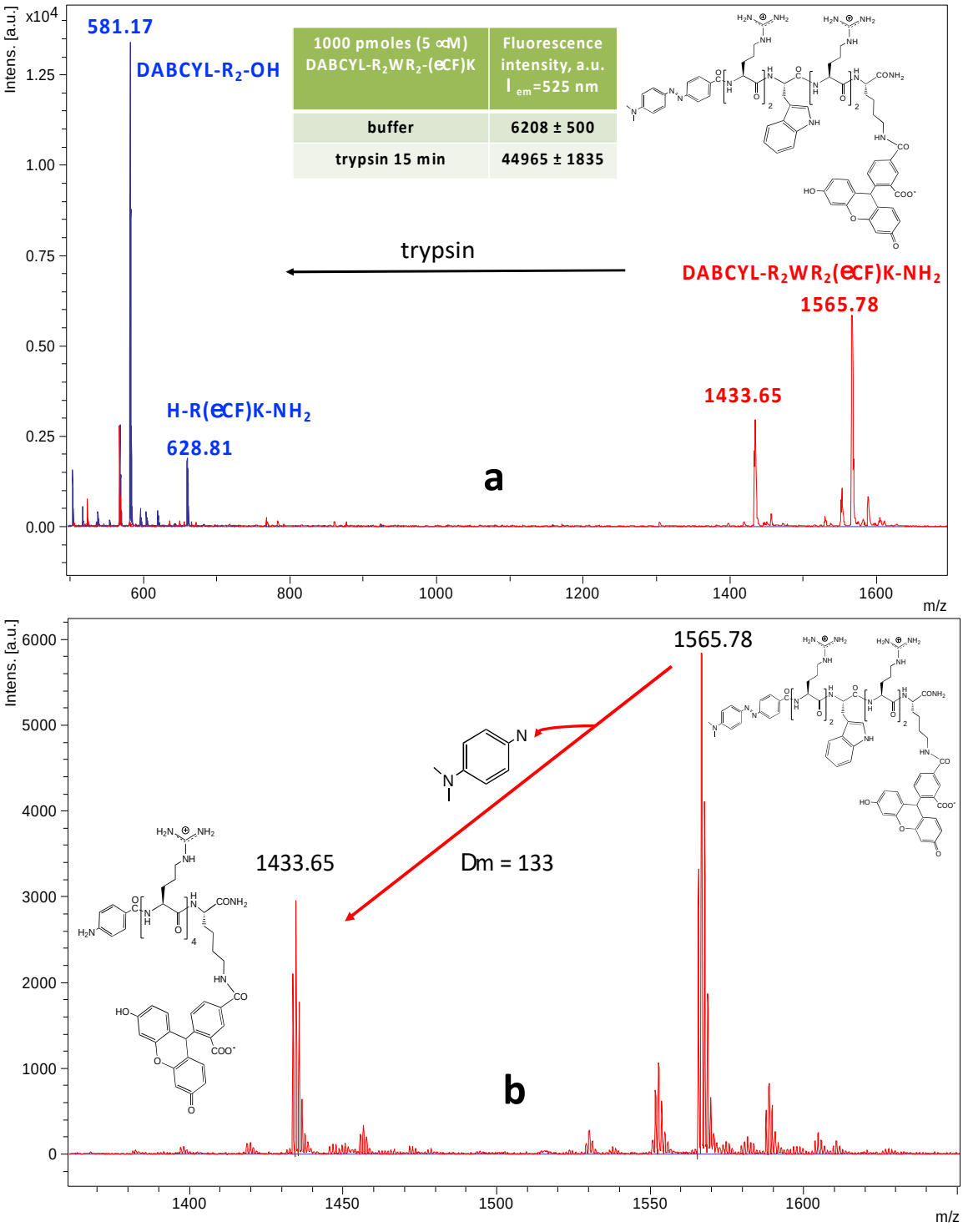


Figure S4. MALDI-ToF mass spectrometry and fluorescence intensity (λ_{em}=525 nm) of DABCYL-R_2WR_2-(εCF)K treated or not with trypsin. (**a**) the MS spectrum in red corresponds to the intact peptide (1000 pmol, $[M+H]^+$ at m/z 1565.78 and 1433.65) in buffer; in blue the MS spectrum after incubation of the peptide (1000 pmol), with trypsin (0.0008%); the table shows the fluorescence signal obtained from the same samples; (**b**) in-source fragmentation observed on the DABCYL moiety, leading to the peptide loss of Δm = 133.