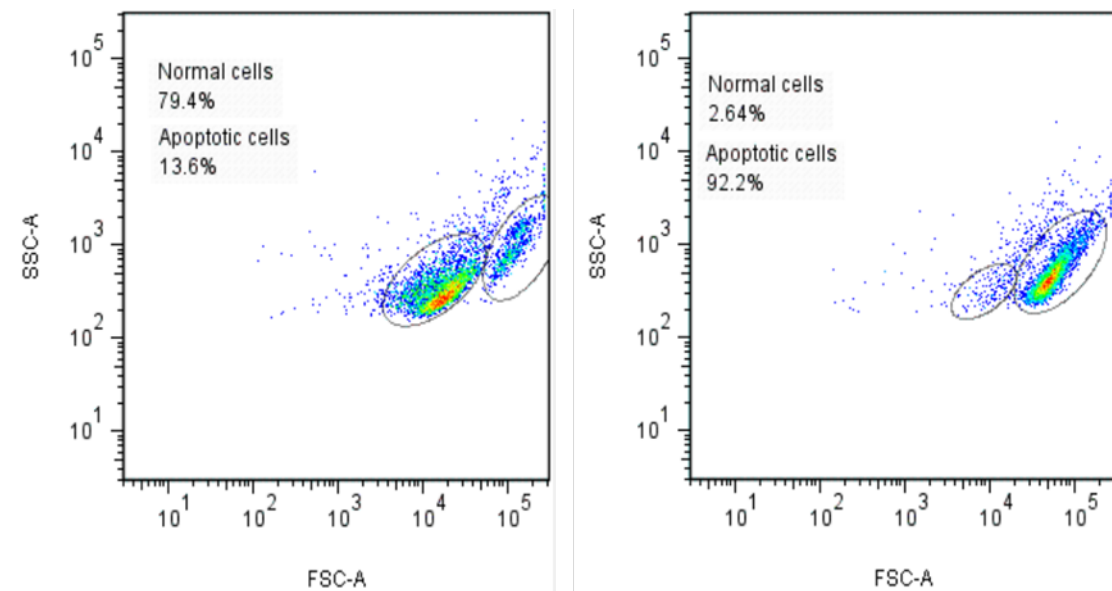


Quantification of caspase-3 in apoptotic cells by flow cytometry

Localisation of apoptotic cells by flow cytometry. Flow cytometry analysis of morphology (SSC-A) and size (FSC-A) of HEK293T cells without (left image) and with (right image) treatment with 1 μ M staurosporine for 4 h.



Calculation of active caspase-3 concentration inside cells

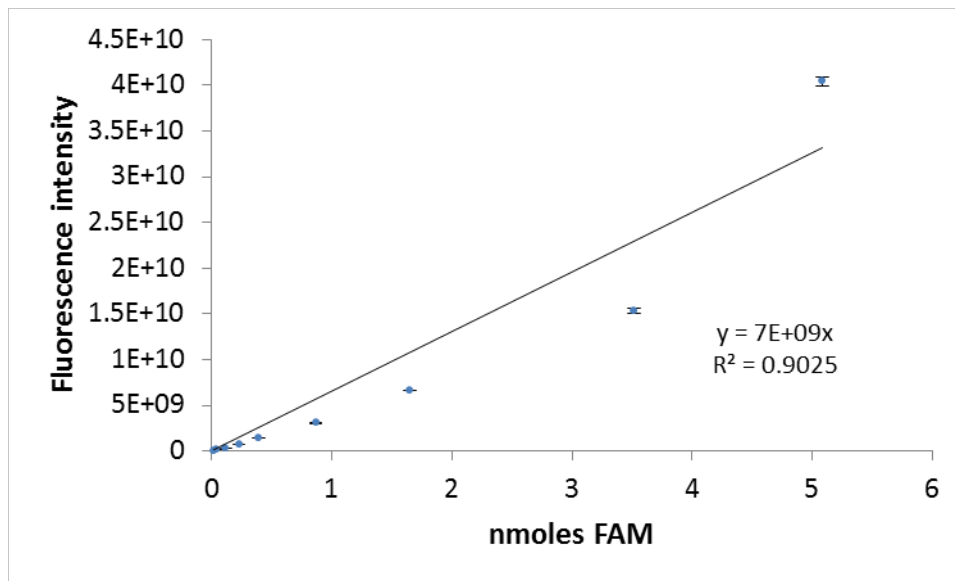
Substrate **27** was used to quantify caspase-3 per cell.¹

1. Calculate FAM-Ahx-Leu-Pro-Asp released per cell:

Fluorescein-labelled microspheres (0.5% SC, ≈ 2 mmol/g),² were used to generate the standard curve for fluorescence intensity versus amount of the fluorophore (nmol) by flow cytometry (see below).

¹ Kossler, S.; Nofziger, C.; Jakab, M.; Dossena, S.; Paulmichl, M. *Toxicology*, **2012**, *292*, 123-135.

² a) A. uncit-Broceta, E.M.V. Johansson, R.M. Yusop, R.M. Sanchez-Martin and M. Bradley, *Nat. Protocol*, **2012**, *7*, 1207-1218. b) J.M. Cardenas-Maestre and R.M. Sanchez-Martin, *Org. Biomol. Chem.*, **2011**, *9*, 1720-1722.



FAM standard curve to determine conversion factor (7×10^9 MFI/nmol) representing FI (Fluorescence Intensity) versus FAM quantity (nmol).

To determine the concentration of FAM-Ahx-Leu-Pro-Asp released per cell (nmol/cell)
 $= [FI/\text{cell} - FI_0/\text{cell}] / 7 \times 10^9 \text{ FI/nmol}$

Assuming 20 μm cell diameter and 2/3 of that for the nucleus, the cytoplasmic volume of HEK293T cell is 3 pL (VC).^{1,3}

FAM-Ahx-Leu-Pro-Asp released per cell (M) = FAM-Ahx-Leu-Pro-Asp released per cell (fmol/cell)/3000 fL

2. Calculate the rate of product formation V_0 with 5 h staurosporine treatment:

$V_0 = d[P]/dt = \text{FAM-Ahx-Leu-Pro-Asp released per cell (mM)} / 18000 \text{ sec}$

3. Quantify the concentration of the enzyme per cell at high substrate concentrations (enzyme saturation)

$$k_{cat} = V_{max}/[E]$$

$$k_{cat} = 0.04 \text{ s}^{-1} \text{ (sensor 27 DLPDK)}$$

[E] = enzyme concentration per cell (nM)

[Caspase-3] = $15.65 \pm 0.46 \text{ nM}$ (1.3 ng active caspase 3 per 10^6 apoptotic staurosporine-treated HEK293T cells).⁴

³ Y. Liu, X. Yan and T. Zhou, *PLOS one*, 2013, 8, 8.

4. Quantify the number of molecules per cell of caspase-3

$N^{\circ}E$ (number of enzyme molecules per cell) = $[E] \times V \times C \times N_A$

$N_A = 6.023 \times 10^{23}$ molecules/mol

$N^{\circ} \text{Cas3} = 28271.22$ molecules/cell

Sensor 27	fmol FAM/cell	FAM (mM)	V (mM)/min	[E] (nM)	E molecules/cell
Average	0.029571429	0.00766667	0.009857143	15.65	28271.22
SD \pm	0.000868966	0.00022529	0.000289655	0.46	830.76

⁴ Roff, C. F. *et al*, *Anal. Biochem.* **2000**, *284*, 114-124.