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DL18: Development of a real-time measurement system for the assessment of caspase-8/-10 activation

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Royal College of Surgeons Ireland (RCSI) (Beneficiary 18)

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Dissemination Level		
PU	Public	PU
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

Description of DL18 with completion date “Month 12”

Editor: Jochen Prehn, Marcus Rehm and Heinrich Huber

Development of a real-time measurement system for the assessment of caspase-8/-10 activation

Single cell measurements using Fluorescence Resonance Energy Transfer

Live cell epifluorescence and confocal imaging based of Fluorescence Resonance Energy Transfer has become as powerful tool for studying real time signaling kinetics within single living cells. As cells within a population do not commit apoptotic cell death synchronously, single cell analysis provide a realistic picture of intracellular signal transduction when compared to bulk studies which yield kinetics representing population averages. As one of the internationally leading research groups in the field of real time analysis of cell death signaling, the Department of Physiology and Medical Physics at the Royal College of Surgeons in Ireland, has delivered to the APO-SYS platform a novel probe for single cell measurements of the activation and activity of initiator caspases-8/-10 [4]. The probe serves to measure signaling kinetics in the extrinsic pathway (WP8). Likewise, as time lapse single cell microscopy was shown as ideal complement to models of signal transduction pathways, the FRET probe will allow the generation of data which can be directly associated with kinetics systems models of the extrinsic apoptosis pathway.

Establishment of the caspase-8/-10 FRET probe

A DEVDase responsive enhanced CFP-DEVD-Venus FRET cassette was obtained from pSCAT3 (a generous gift of Masayuki Miura, RIKEN Brain Research Institute, Wako, Saitama, Japan) and subcloned into pTK-RL (Promega, WI). The resulting plasmid was digested to remove the DEVDase substrate cassette and ligated with excess annealed oligonucleotides, forming an IETDase substrate cassette, providing an optimized recognition site for caspases-8/-10. The resulting pTK-reverse SCAT8 vector was digested with BamHI and HindIII to obtain the enhanced CFP-IETD-Venus FRET cassette. This was ligated into pcDNA3.1 vector to generate the final vector product pSCAT8. The probe can be transfected into any eukaryotic cell line and positive clones can be selected based on neomycin selection.

Probe validation and applicability

- Sensitivity and signal to noise ratio: The uncleaved FRET probe was found to exhibit exceptionally strong resonance energy transfer, estimated to be at least 10 times more efficient than classical protein-protein FRET pairs. Upon reduction of measurement noise, the optimized SNR provided sensitivities of substrate cleavage in the low percentage range.
- Fluorescence characteristics: Strong emission peaks for CFP, FRET and Venus channels can be clearly separated with optimized filter and beamsplitter settings to minimize channel crosstalk. Full details for the spectral characteristics of the optical components and manufacturers can be provided to APO-SYS platform partners.
- Multiplexing possibilities: The FRET probe is spectrally compatible with other widely used fluorophores in red-shifted wavelength range. These include all red fluorescence protein (RFP) generations and RFP-fusion proteins as well as widely used synthetic dyes. These for

example include Ca^{2+} -indicators such as FuraRed, indicators for mitochondrial bioenergetics such as tetramethylrhodamine-methylester, nuclear stains such as Draq5 and a multitude of other commercially available indicators as those listed in online repositories such as <http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook.html>

Experimental measurements:

We investigated the signalling dynamics during apoptosis initiation via the TRAIL receptor pathway to investigate how variability in drug exposure can be translated into a cell death execution decision. FRET-based microscopy demonstrated dose-dependent responses of caspase-8 activation and activity within individual living HeLa cells. Caspase-8 on average was activated 45 to 600 min after TRAIL addition. Caspase-8-like activities persisted for 15 to 60 min before eventually inducing mitochondrial outer membrane permeabilisation (MOMP). Independent of the TRAIL concentrations used or the resulting caspase-8-like activities, MOMP was induced when 10 % of the FRET substrate was cleaved. In contrast, in Bid-depleted cells caspase-8-like activity persisted for hours without causing immediate cell death. Our findings so far already provided detailed insight into the intracellular signalling kinetics during apoptosis initiation and identified a threshold mechanism controlling the induction of apoptosis execution [4]. We further elucidated the spatiotemporal coordination of caspase-8 induced MOMP [3]. Another manuscript describing the molecular control of the cell death decision threshold based on caspase-8 FRET measurements is currently in preparation [5].

APO-SYS related publications from RCSI:

1. Hector S, Prehn JH. - *Biochim Biophys Acta*. 2009 Jan 6. [Epub ahead of print]
2. Huber HJ, Plchut M, Weisová P, Dössmann H, Wenus J, Rehm M, Ward MW, Prehn JH. - *J Neurosci Methods*. 2009 Jan 30;176(2):270-5.
3. Rehm M, Huber HJ, Hellwig CT, Anguissola S, Dussmann H, Prehn JH. - *Cell Death Differ*. 2009 Jan 9. [Epub ahead of print]
4. Hellwig CT, Kohler BF, Lehtivarjo AK, Dussmann H, Courtney MJ, Prehn JH, Rehm M. - *J Biol Chem*. 2008 Aug 1;283(31):21676-85
5. Hellwig CT, 2009 in preparation