Fluorescent peptides as molecular probes

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Fluorescent peptides, peptides with a reporter fluorescent dye, are valuable probes used in visualising intracellular processes and molecular interactions at the level of single cells.[1] The fluorescent dye can be attached to the amino (N) or carboxy (C) terminus, or in the case of FRET (fluorescence or Förster resonance energy transfer) peptides, the two dyes (donor and acceptor) can be at the amino or carboxy termini or in the internal peptide sequence. Some of the advantages of using fluorescent peptides include higher sensitivity because of the fluorescent probe,[1] better specificity because of the amino acid sequence,[1] and, where radiolabeled peptides are used, less concern with radioactive waste disposal.[2] This article features the different fluorescent peptides that AnaSpec has manufactured, their uses and applications.

For *in-vivo* imaging

Fluorescent peptides can be used for *in-vivo* studies. In *in-vivo* imaging, three-dimensional fluorescence images of the internal structures, especially of small animals, are produced. This technique requires the use of near infrared red (NIR) dyes, since at higher wavelength tissues do not absorb or scatter photons as strongly as when lower-wavelength dyes are used.[3,4]

Extracellular matrix proteins that contain the RGD (Arg-Gly-Asp) sequence and the integrin receptors that bind this sequence constitute a major recognition system for cell migration and adhesion processes.[5] In a collaboration with J. Rey of the University of South Florida, AnaSpec synthesised a cyclic peptide containing an RGD motif (RGDyK), with AnaSpec's proprietary HiLyte Fluor™ 750 dye (Ex/Em = 754/778 nm) conjugated to the side chain of lysine. Figure 1 shows the fluorescent peptide preferentially binding to certain organs that are known to be rich in integrin $\alpha v\beta 3$. Through *in-vivo* imaging, the progression of the clearance of the peptide-dye conjugate was monitored over a period of 48 hours (data not shown). To view more results of this study, access the Webpage listed in the reference section below.[6]

For enzyme activity detection

Peptide substrates used in detection of enzyme activity can contain either a single dye or, in the case of FRET, two dyes. In the intact fluorescent substrates there is low fluorescence prior to enzyme cleavage. Upon recognition of the substrate by a specific enzyme and subsequent cleavage, the quenched fluorescence is recovered. Increase in fluorescence is correlated to enzyme activity.[4] Using a fluorimetric plate reader, continuous assay of enzyme activity and high-throughput screening (HTS) of enzyme inhibitors can be performed.

Single-dye labeled peptides, mostly C-terminal dye labels, provided by AnaSpec include substrates for caspases, cathepsin, HDAC (histone deacetylase), calpain, kallikrein, and others. Dyes used in these substrates include dyes in the blue range (AMC, AFC), green range (Rh110) or in the red range (AnaRed™). One major advantage of using longer-wavelength dye-labeled

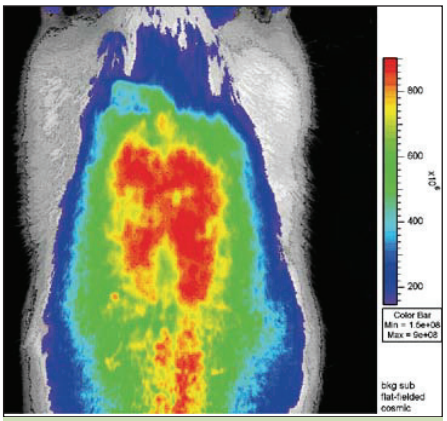

Fig 1. In-vivo image of a rat injected with 20nmol of c[RGDyK(HiLyte Fluor 750)] peptide-dye conjugate 3 hours post injection (image courtesy of J. Rey, University of South Florida).

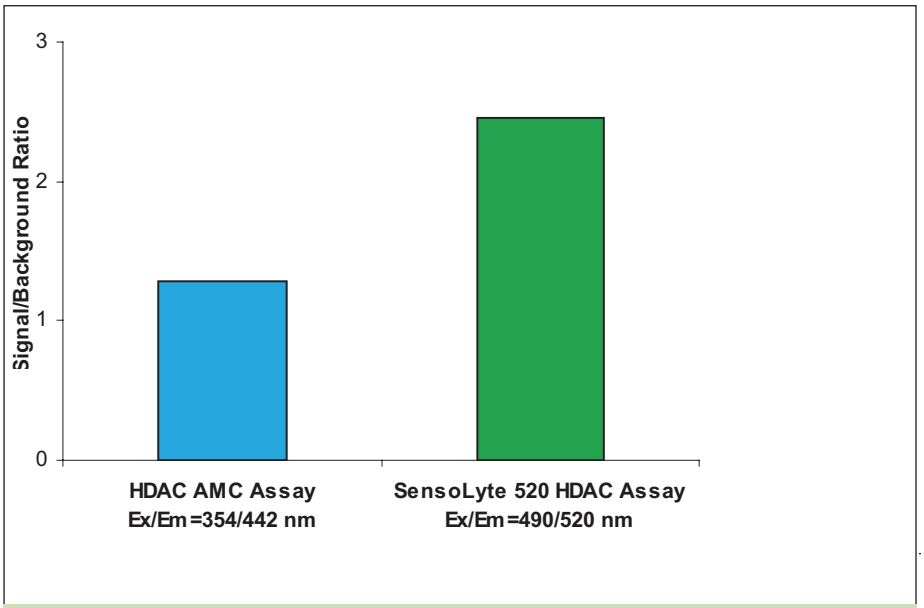

Fig 2. The SensoLyte® 520 assay kit shows less interference from cell components and allows detection in Phenol Red containing medium. It is currently the industry's most sensitive HDAC assay kit.

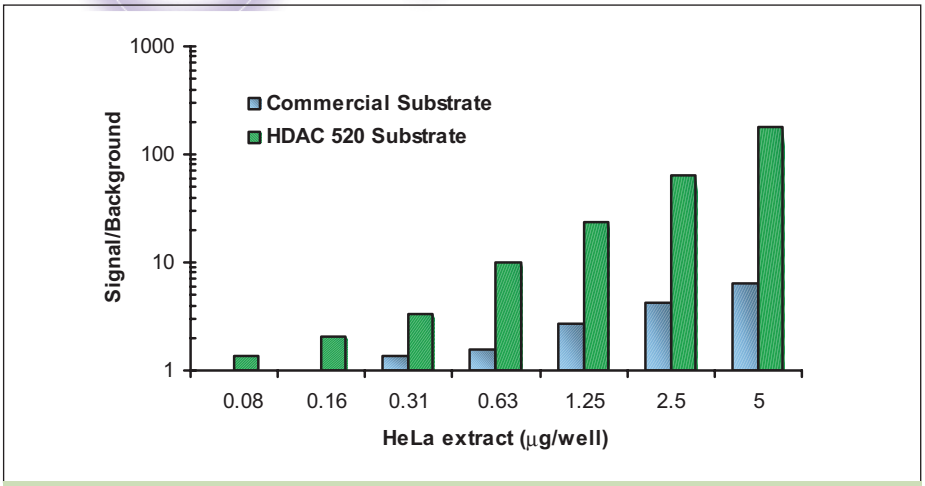

Fig 3. The SensoLyte® 520 assay kit provides higher sensitivity and better linear range than the AMC substrate. 25 mM HDAC substrates were incubated with HeLa nuclear extracts followed by a 15 min incubation with a Trichostatin A-containing developer.

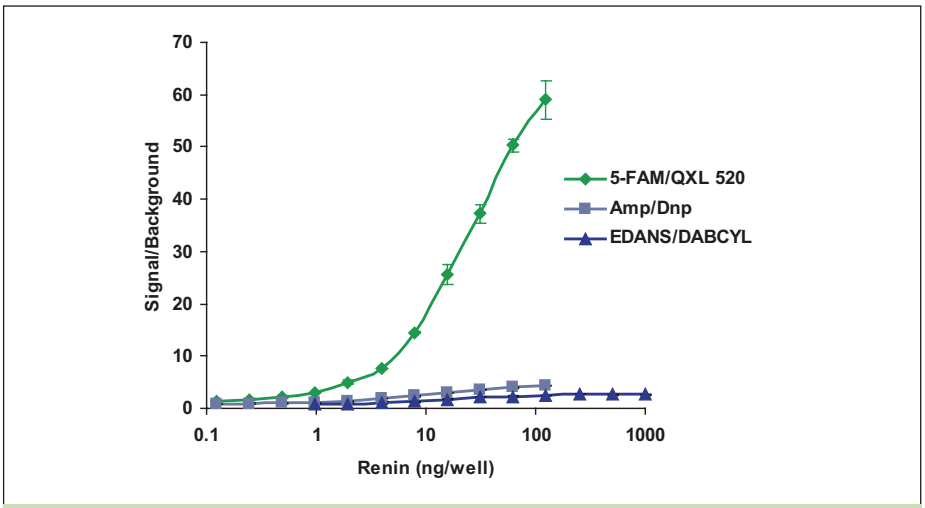

Fig 4. Comparison of three renin assay kits.

peptides is that autofluorescence from cellular components is minimised. In a side-by-side comparison of an existing blue-range HDAC substrate (AMC) and a green-range HDAC substrate developed by AnaSpec and used in the SensoLyte® 520 HDAC Assay Kit, the longer-wavelength substrate shows significantly higher sensitivity than the AMC substrate (Figs 2 and 3).

FRET peptides are peptides that contain a donor (fluorophore) and an acceptor (another fluorophore or a quencher). The donor molecule is usually placed on one terminus and a quencher molecule on the other terminus, separated by a peptide containing the protease cleavage site. In some instances, the donor and acceptor molecules can be placed in the internal sequence as well. FRET is a distance-dependent transfer of excited-state energy from an initially excited donor to an acceptor, with the donor molecule typically emitting at shorter wavelengths that overlap with the absorption of an acceptor.[7] FRET occurs when a donor and an acceptor are within a specified distance, usually 10-100Å. Within this distance, the fluorescence of the donor is quenched. Enzyme hydrolysis of the peptide results in spatial separation of the donor and acceptor, which leads to the recovery of the donor's fluorescence.[7] Utilising a novel FRET pair, 5-FAM/QXL™ 520, AnaSpec has developed a series of long-wavelength protease substrates and assay kits that can be used in drug discovery. Compared to the existing substrates using the traditional EDANS/DABCYL FRET pair, these 5-FAM/QXL™ 520 substrates exhibit better sensitivity because: (1) The absorption spectrum of QXL™ 520 overlaps with almost the entire emission spectrum of 5-FAM, thereby providing efficient quenching; (2) The hydrophilicity of QXL™ 520 results in better solubility of the peptide substrate; (3) The extinction coefficient of 5-FAM is 13-fold higher than EDANS; (4) The longer wavelength fluorescence of 5-FAM is less interfered by the autofluorescence and absorbance of drug candidates and cellular components.

The SensoLyte® 520 Renin assay kit employs a 5-FAM/QXL™ 520 FRET pair containing substrate. This substrate exhibits femtomole sensitivity – the highest sensitivity available for any commercial renin assay kit. It is 40x more sensitive than the leading competitor's (Fig 4). Other long-wavelength protease assays developed by AnaSpec include substrates for HCV, HIV, WNV; MMPs; α and β-secretases; cathepsin D and S; and aggrecanase-1 (ADAMTS-4).

AnaSpec's SensoLyte® 520 MMP assay kits also employ the 5-FAM/QXL™ 520 FRET pair. These kits are also robust and highly sensitive. They are ideal for screening of MMP inhibitors and for quantitative detection of MMP activity. Another application where the long-wavelength MMP substrates have been used is what Dr Bryan Crawford at the University of New Brunswick has termed 'Differential *in-vivo* zymography.' Two FRET peptides, one containing the 5-FAM/QXL™ 520 FRET pair and the other a 5-TAMRA/QXL™ 570 pair were used to study the differential breakdown of MMP substrates in zebrafish embryos (Fig 5).

For fluorescence microscopy

As mentioned above, fluorescent peptides have been used in visualising cellular processes. AnaSpec was able to track the uptake of dye-labeled CPP using fluorescence microscopy. CPPs (cell penetrating peptides), also known as protein transduction domains (PTDs), are carriers with small peptide domains that can freely cross cell membranes. Several CPPs have been identified that allow a fused protein to efficiently cross cell membranes in a process known as protein transduction. Using a green dye (FITC) labeled Antennapedia and a red dye (TAMRA) labeled TAT peptide (derived from the HIV TAT protein), it was found that these peptides were able to enter HeLa cells (Fig 6).

In Alzheimer's diseased (AD) brain, it has been found that β-amyloid peptides, 40 to 42 amino acids in length, are major constituents of plaques; and Alzheimer's researchers have found different uses for fluorescent β-amyloid peptides. Using a TAMRA labeled β-amyloid (1-42), (Ex/Em = 542/568 nm), Chafekar *et al* showed its uptake by cells as well as its

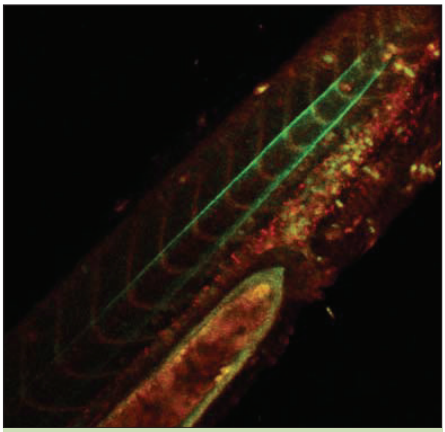

Fig 5. A mixture of QXL™ 570/5-TAMRA-labeled Peptide I incorporating a red fluorophore, and QXL™ 520/5-FAM-labeled Peptide XIII incorporating a green fluorophore were used to detect differential breakdown of the two different MMP substrates in zebrafish. Peptide I was broken down mostly in the maturing myotome boundaries, whereas Peptide XIII was broken down much more strongly in the notochord sheath (courtesy of Dr Bryan Crawford, University of New Brunswick, Fredericton, New Brunswick, Canada).

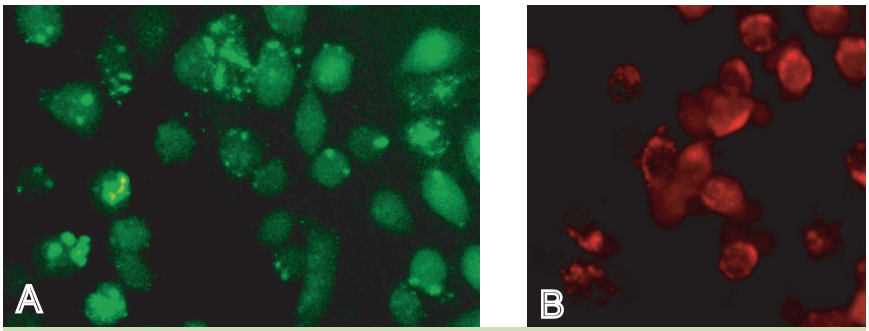

Fig 6. Uptake of CPP by HeLa cells. HeLa cells were incubated with OptiMEM medium containing 10 mM FITC-LC-Antennapedia, cat# 24175, 24176 (panel A) and 10 mM of TAMRA-labeled TAT (47-57), cat# 61211(B) for 1 hour, washed and analysed by fluorescence microscopy.

subcellular localisation using confocal microscopy and immunofluorescence microscopy.[8] Vestergaard *et al* tracked the movement of HiLyte Fluor™ 488 labeled β-amyloid (1-42) at various aggregation states in real time.[9] In a study by Hickman *et al* HiLyte Fluor™ 488 labeled β-amyloid (1-42) was incubated with TNFα-treated or untreated N9 cells and cell-associated fluorescence was monitored by flow cytometry.[10]

AnaSpec has an extremely broad array of fluorescent dye labeled β-amyloid peptides. Labeling options include a full spectrum of fluorescent dyes, ranging from classic dyes like FAM and TAMRA to AnaSpec's proprietary HiLyte Fluor™ series of high-performance dyes (see Table 1). A suite of N- and C-terminally biotin-labeled β-amyloid peptides is also available.

The above highlights a few of the applications for fluorescent peptides. Fluorescent peptides have also been used in fluorescent polarisation studies, as cross-linking imaging probes,[4] and others. It is evident that the uses for these powerful research tools will become even more sophisticated with the advancement of better reagents and equipment. *sp²*

Dye	Ex/Em (nm/nm)	beta-Amyloid (1-28)	beta-Amyloid (1-40)	beta-Amyloid (1-42)
AMCA	354/442	+	+	+
5-FAM	494/521	+	+	+
FAM-Aβ-Biotin	494/528		+	
Hilyte Fluor™ 488	503/528	+	+	+
HiLyte Fluor™ 555	551/567	+	+	+
TAMRA	544/572	+	+	+
HiLyte Fluor™ TR	584/607	+	+	
HiLyte Fluor™ 647	649/674	+		

Table 1. Examples of N-terminally labeled fluorescent β-amyloid peptides. C-terminal dye-labeled β-amyloid peptides are also available. The colour shading corresponds to the colour of the emission wavelength.

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