FRET-Based Assays for the Detection of Amyloid Degrading Protease Activity
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Introduction
Amyloid degrading proteases (ADPs) are major players in β-amyloid (Aβ) clearance. The accumulation of Aβ plaques, a hallmark of Alzheimer’s disease (AD) patient brain, has been hypothesized to be due to the imbalance between ADP’s activity and Aβ production, resulting in AD pathogenesis. In order to confirm the hypothesis of ADPs in neuronal degeneration diseases, reliable assays for ADPs activity detection have to be developed.

We have designed a panel of assays to detect ADP targets using FRET ( Förster resonance energy transfer)-based peptide substrates. To develop assays for ADAM10, TACE (ADAM17, a metalloproaposite, Neprilysin and IDE (a Cu/Zn degrading enzyme), we synthesized FRET substrate peptides labeled with the fluorophore, 4-carboxyfluorescein (4FAM) and the quencher, QXL520. The fluorescence of 4FAM is quenched by QXL520 and recovered upon cleavage of the peptide by active ADP. Fluorescence is then monitored at the excitation/emission wavelengths of 495/520 nm.

Many of ADPs targets require low pH for optimal activity, and 5FAM shows decrease of signal in acidic environment. To optimize FRET substrates for maximum sensitivity at low pH, we introduced a new pH-independent Biocluorophore, HiLyte™ Fluor 488 in place of 5FAM. Besides unquenched signal at low pH, HiLyte™ Fluor 488 has excellent brightness at the same wavelength as 5FAM. The absorbance spectrum of QXL520 overlaps with the emission spectrum of HiLyte™ Fluor 488, providing optimal coquenching. Substrates with HiLyte™ Fluor 488/QXL520 were used for detection of β-secretase, cathepsin B and D, providing higher sensitivity and better assay window.

Some of ADPs, such as MMP-2 and MMP-9, belong to matrix metalloproaposites that are known for overlapping substrate specificities. To avoid non-specific substrate cleavage by multiple proteases, we utilized an Immunocapture technique in addition to the FRET assay. Monoclonal antibody were applied to pull down relevant MMP from biological sample and subsequent addition of MMP substrate peptide containing the 5FAM/QXL520 FRET pair. These assays provided significant increase of specificity of MMP-2 and MMP-9.

Materials and Methods
- Sensory™ 520 ADAM10, TACE (ADAM17), Neprilysin and IDE Assay Kits
- FRET 5FAM/QXL520 substrate, ExEm=495/520 nm upon cleavage
- Sensory™ 520 BACE, Cathepsin B and Cathepsin D Assay Kits
- FRET HiLyte™ Fluor 488/QXL520 substrate, ExEm=495/520 nm upon cleavage
- Sensory™ Plus 520 MMP-2 and MMP-9 Assay Kits
- FRET 5FAM/QXL520 substrate, ExEm=495/520 nm upon cleavage
- Anti-MMP MAb, recombinant MMPs and culture supernatants containing MMPs
- Sensory™ 520 assay activity assay
- Protocol fixation volumes were 50 µl of purified enzyme sample and 50 µl of FRET substrate solution. Assays were performed in 96-well black plate
- Immunocapture MMP Plus assay activity assay
- Protocol fixationsamples containing MMPs were activated with 1 mM APMA at 37°C for 1 h. After fixation the 12 x 80-mm strip plate was preincubated with antibody. After 2 h incubation at room temperature for Immunocapture, plate was washed and 5FAM/QXL520 FRET peptide substrate was added and incubated for 1-3 h at room temperature.
- Fluorescence measurement. FlexStation 3844 (Molecular Devices, Sunnyvale, CA)

Conclusions
- We have developed a series of FRET-based assays to detect the activity of ADPs, such as ADAM10, TACE, 4-carboxy fluorescein, IDE, Neprilysin, Cathepsins B and D, MMPs-2 and -9. These assays are capable of measuring sub-nanogram range of enzymes.
- High sensitivity of assays was achieved using proprietary FRET pairs, 5FAM/495-QXL520 and HiLyte™ Fluor 488-QXL520. The latter FRET pair provided stable fluorescent signal at acidic pH resulting in assay optimization for cathpsins and neprilysin that require low pH for maximum activity.
- To differentiate between proteases activities in biological samples, we combined FRET and ELISA principles. These FRET-based immunocapture assays enables detection of specific matrix metalloproaposites, such as MMP-2 and MMP-9, which are involved in amyloid degradation.