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*Original Article*

# Establishing a cellular FRET-based fluorescence plate reader assay to monitor proNGF-induced cross-linking of sortilin and the neurotrophin receptor p75$^{NTR}$

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**Abstract:** Whereas the proform of the nerve growth factor (proNGF) is crucial for eliminating superfluous cells during neuronal development it also promotes apoptosis following brain trauma and neuronal injury. The apoptotic signal is elicited upon formation of a trimeric receptor complex also containing the vps10p domain receptor sortilin and the neurotrophin receptor p75$^{NTR}$. However, proNGF-induced receptor complex formation has been difficult to directly assess other than by western blotting. We here describe a fluorescence resonance energy transfer (FRET) based fluorescence plate reader assay to monitor the interaction between fluorescently tagged sortilin and p75$^{NTR}$ in live cells. The method is based on a standard fluorescent plate reader found in many biochemical laboratories and the results are evaluated using a microscopy-based quantified sensitized acceptor emission FRET approach making use of a pair of FRET standard constructs. As a result, the effect of proNGF on the interaction between sortilin and p75$^{NTR}$ can be evaluated in live cells allowing for screening and selection of therapeutic compounds interfering with proNGF-induced cell death.

**Keywords:** FRET-based fluorescence plate reader assay, sortilin, p75$^{NTR}$, proNGF, high throughput

## Introduction

The nerve growth factor (NGF) belongs to the family of growth factors of the neurotrophin family and promotes neuronal survival through the transmembrane neurotrophin receptor p75$^{NTR}$ in conjunction with the tropomyosin receptor kinase A (TrkA) [1, 2]. However, NGF is synthesized as a proform (proNGF) with opposing biological activity to that of its mature counterpart as it promotes apoptosis rather than survival [3-5]. This is achieved by engaging in a heterotrimeric receptor complex also containing p75$^{NTR}$ and the neuronal transmembrane receptor sortilin a member of the vps10p domain receptor family [6]. Whereas sortilin engages the prodomain of proNGF, p75$^{NTR}$ targets the mature part of the molecule thereby increasing the affinity for proNGF approximately 100-fold compared to that for each receptor alone [6]. However, sortilin and p75$^{NTR}$ also form a protein complex in the absence of ligand, as they interact through the extracellular juxtamembrane domain of p75$^{NTR}$ [7]. Whereas proNGF promotes neuronal apoptosis during development as demonstrated for postmitotic retinal ganglia cells in the mouse retina [8] proNGF also promotes apoptosis in animal models following neuronal injury such as after kainic acid-induced seizures [9] and following sciatic nerve transection [10]. The proform of other neurotrophins i.e. brain derived neurotropophic factor (proBDNF) and neurotrophin-3 (proNT-3) likewise promote apoptosis by complexing p75$^{NTR}$ and sortilin [11-13].

Using live cells, we aimed at developing a high throughput assay with a standard fluorescent plate reader as detection instrument to monitor the interaction between fluorescently tagged sortilin and p75$^{NTR}$. FRET occurs when a donor fluorophore transfers energy to an acceptor flu-

orophore with the FRET efficiency being strongly dependent on the proximity of the two fluorophores, which increases rapidly as the distance between the fluorophores is reduced from 8 to 2 nm. The development of new variants of the green fluorescent proteins has facilitated the use of these proteins in FRET to indicate the proximity of tagged proteins. A well-described FRET pair is the fluorescent proteins cerulean [14] and venus [15], which have better spectral properties in terms of FRET compared to the previously used YFP and CFP [16].

In this paper, we describe the use of a standard fluorescent plate reader in a fluorescent FRET-based assay using live cells expressing the fusion proteins sortilin-cerulean and p75$^{NTR}$-venus. We show how this system can be used to evaluate the effect of proNGF on the interaction between sortilin and p75$^{NTR}$. The FRET based assay may provide a new tool for screening potential compounds inhibiting proNGF-induced apoptotic cross-linking of sortilin and p75$^{NTR}$.

## Material and methods

*DNA work*

A PCR-mediated overlap extension strategy was employed to generate sortilin-cerulean fused via a five amino acids linker (TWQRR). Using sortilin$^{mut}$/pcDNA3.1/Zeo(-) [7] as template, the primers 5'-CCAGGGGACAAATGCCAGGG-3' and 5'-ACCATTCTCCGCTGCCAGGTCACAATGAGCACTCCTGCTAC-3' were used to amplify the upstream fragment. Likewise, using cerulean-C1 [14] as template, the primers 5'-TTGTGACCTGGCAGCGGAGAATGGTGAGCAAGGGCGAGGAG-3' and 5'-CACACACTTAAGCTACTTGTACAGCTCGTCCATGCC-3' were used to amplify the downstream fragment. The overlapping PCR products were amplified and inserted into sortilin$^{mut}$/pcDNA3.1(-)/Zeo, taking advantage of the sortilin luminal BSPEI site and the primer generated AflII site. A similar strategy was used to generate HA-p75$^{NTR}$-venus. For amplification of the upstream fragment, rat HA-p75$^{NTR}$/pcDNA3.1/G418 was used as template together with the primers 5'-CACACAGCGGCCGCACCATGTCTGCACTTCTGATC-3' and 5'-ACCATGCTGTTCCACCTCTTGAAAGCAATATAGGCCACAAG-3'. For the downstream fragment, venus-pcs2 [15] served as template and primers were 5'-AAGAGGTGGAACAGCATGGTGAGCAAGGGCGAGGAG-3' and 5'-CACACAGAATTCTTACTTGTACAGCTC-3'. The final fragment was inserted into pcDNA3.1(-)/G418 (Invitrogen) using the primer-generated NotI and EcoRI site.

The FRET standard C5V, with cerulean and venus directly tethered by an amino acid linker of five amino acids, has previously been described [17]. The CTV FRET standard, with cerulean and venus separated by 229 amino acids is described in [18].

*Cell lines and culturing*

Human embryonic kidney cells (HEK293) and rat schwannoma cells (RN22 cells) were cultured and stable cell lines generated as described in [7]. Identification of positive clones was done by western blotting and fluorescent microscopy.

*Western blotting*

Stably transfected HEK293 cells were processed and subjected to western blotting as previously described [7]. Primary antibodies were anti-NTR-3 (612100) from BD Transduction Laboratories, anti-HA (H6908) and anti-β-actin (A5441) from Sigma.

*FRET plate reader data acquisition and analysis*

HEK293 cells stably co-expressing sortilin-cerulean and p75$^{NTR}$-venus or cells expressing one of the two fusion proteins alone as well as untransfected cells (for background fluorescence) were seeded into Corning black 96-well microtiter plates (Sigma) at a density of 20,000/well in triplicates two days prior to the experiment. Immediately before the experiment, medium was exchanged with 200 µl PBS containing 1 mM $CaCl_2$ and $MgCl_2$. Recombinant human furin cleavage-resistant proNGF was then added directly to wells and measurements performed after 30 min at 20°C on a fluorescent plate reader (Victor3, 1420 Multilabel counter, Perkin Elmer). CW lamp energy was set at 10,000 units with continuous lamp control. Counting times were 0.1 sec and a large aperture and readings from the bottom of the well was used. The following filter set was used: cerulean filter set (excitation: 430/15 nm, emission: 460/20 nm); venus filter set (excitation: 485/15, emission 535/15); FRET filter set (430/15 nm, emission 535/15).

Calculation of the apparent FRET efficiency ($E_{app}$%) was performed as follows: Average background values were calculated for each of the three channels and subtracted from the measurements.

The raw FRET signal obtained from the plate reader is contaminated by overlap of the donor emission spectrum with that of the acceptor (donor spectral bleed through (DSBT)) and by direct excitation of the acceptor by the donor excitation light (acceptor spectral bleed trough (ASBT)). This contamination must be quantified and subtracted from the raw FRET signal.

$$DSBT = CV_{DexDem} \times \frac{C_{DexAem}}{C_{DexDem}} \text{ and}$$

$$ASBT = CV_{AexAem} \times \frac{V_{DexAem}}{V_{AexAem}}$$

Where CV represents cells expressing sortilin-cerulean and p75$^{NTR}$-venus, C is cells with expression of cerulean, and V is cells with expression of venus. Dex is donor excitation light, Dem is donor emission signal, Aex is acceptor excitation light and Aem is acceptor emission signal.

The corrected FRET signal is:

$$Fc = raw\ FRET\ signal \mid ASBT \mid DSBT$$

Where Fc is the corrected FRET value. The apparent FRET efficiency is calculated as:

$$E_{app}\% = 100 \times \frac{Fc}{Fc + CV_{DexDem}}$$

*Confocal laser scanning microscopy and FRET analysis in live cells*

HEK293 cells with a stable expression of sortilin-cerulean and p75$^{NTR}$-venus were seeded two days prior to imaging onto poly-L-Lysine (Sigma) coated coverslips. To assess the effect of proNGF, medium was exchanged with pre-heated PBS (with 1 mM $CaCl_2$ and $mgCl_2$) with and without addition of proNGF and incubated 45 min at 37°C. Imaging was performed on a Zeiss LSM510 confocal microscope equipped with a 40X C-Apochromat objective, N/A 1.2. The laser lines 458 nm and 514 nm were used for excitation. Detection was performed using the Meta-detector with a band pass between 469 -501 nm and 533-576 nm.

For quantifying FRET-values, collected images were analyzed using the algorithm implemented in the ImageJ-based PFRET software developed by Professor Periasamy [19]. Using this software, a possible dependence of DSBT and ASBT on donor- and acceptor signal levels are taken into account. All calculations and corrections were performed on background-subtracted images. Lower bounds for signal levels used in ASBT and DSBT correction calculations were set to 15 intensity units. Regions of interest (ROI's) were chosen automatically on the PFRET image and set to 5X5 pixels. ROI's were only included in the calculation for the final $E_{app}$% if the signal levels in the donor, acceptor, and FRET channels were above the lower bound value (25 intensity units). PFRET analysis was only performed on the cell surface as a new plug-in was developed for ImageJ, which enabled us to place a mask on each image leaving only the membrane for analysis.

*FRET analysis of fixed RN22 cells stained with Alexa 488 and Alexa 555 antibodies*

RN22 cells were seeded onto poly-L-Lysine coated coverslips and subsequently incubated with and without proNGF in PBS for 45 min at 20°C. Cells were fixed in 4% paraformaldehyde, blocked in 10% FCS, and incubation with anti-sortilin (#5264) 1:200 (custom made by Dako [20]) and anti-NGFR 1:50 (1157, R&D systems). Secondary antibodies were Alexa-conjugated donkey anti-rabbit 555 (A31572) (1:800) and donkey anti-goat 488 (A11055) (1:500) from Molecular probes. Analysis of cells was done on the Zeiss confocal LSM510 META microscope using a 40x NA 1.2 C-Apochromat. Sensitized acceptor emission FRET measurements and analysis were performed according to the PFRET procedure [19], using the associated ImageJ PFRET plugin. Donor and acceptor signals were detected through 500-530 nm and 565-615 nm emission filters following excitation with 488 nm (donor) or 543 nm (acceptor) using in the range of 10-25 μW and 40-90 μW of laser power, which were kept constant during acquisition of each data set. Using the ImageJ PFRET plugin, a possible dependence of donor- and acceptor spectral bleed throughs (DSBT and ASBT) on donor- and acceptor signal levels are taken into account. Furthermore, it is possible to obtain not only bleed through-corrected FRET-channel images (PFRET images) but also $E_{app}$ images as well as $E_{app}$ values from ROIs. All calculations and corrections were performed on background-subtracted images. Lower bounds for signal levels used in ASBT and DSBT

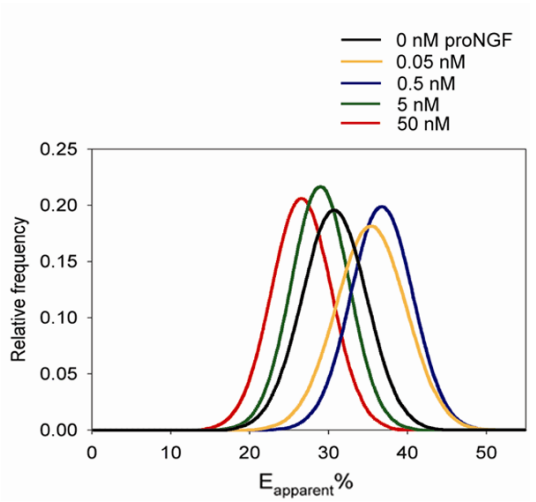


**Figure 1.** FRET analysis of the sortilin - p75$^{NTR}$ interaction in RN22 cells. RN22 cells with endogenous expression of sortilin and p75$^{NTR}$ were incubated with proNGF followed by fixation and staining with anti-sortilin and anti-p75$^{NTR}$ primary antibodies and fluorescently tagged secondary antibodies. Most groups were significantly different compared to untreated cells ($p < 0.001$) except cells treated with 5 nM proNGF (n=10 for all concentrations). All data are presented as the mean ± SD.

correction calculations were set to 15 intensity units. ROI's were chosen automatically on the PFRET image and set to 5X5 pixels. ROI's were only included if the signal levels in the donor, acceptor, and FRET channels were above the lower bound value (20 intensity units) for more than 80% of the pixels within the ROI.

*Statistics*

Assuming unequal variances, RN22 cell data were analyzed by a one-way ANOVA followed by Games-Howell's correction. Furthermore, non-parametric multiple-independent-samples test (Kruskal-Wallis H) was used to confirm the analysis in this study.

Treatment responses in different groups of live cells expressing sortilin-cerulean and p75$^{NTR}$-venus using the confocal microscope were analyzed by ANOVA after the data were log10 transformed. If the analysis of variance revealed significant group differences, a post-hoc test (Scheffe) was carried out to elucidate the pattern of group differences.

Treatment responses in different groups of cells using the multiplate reader were compared by ANOVA. If the analysis of variance revealed significant group differences, a post-hoc test (Bonferroni and Turkey) was carried out to elucidate the pattern of group differences.

## Results

*Cross-linking of endogenous sortilin and p75$^{NTR}$ can be monitored by FRET*

In order to establish a FRET-based fluorescence plate reader assay to monitor sortilin - p75$^{NTR}$ interactions, we took advantage of the rat schwannoma cell line RN22, which express' endogenous levels of sortilin and p75$^{NTR}$ and which undergoes apoptosis in response to low amounts of proNGF [7, 21]. Using fixed RN22 cells, we sought to follow proNGF-induced sortilin - p75$^{NTR}$ complex formation by FRET using fluorescently labelled secondary antibodies. The apparent FRET efficiency ($E_{app}$%) was significantly increased at the cell surface upon addition of 0.05 nM proNGF (~18%) and 0.5 nM (~26%) compared to untreated cells (**Figure 1**) demonstrating that FRET is a valid method for detection of proNGF-induced sortilin - p75$^{NTR}$ cross-linking at low ligand concentrations. Interestingly, by adding 50 nM proNGF, $E_{app}$% was significantly decreased (~14%) compared to untreated cells whereas 5 nM proNGF had no significant effect.

*Monitoring sortilin - p75$^{NTR}$ interactions by FRET using labeled receptor constructs in live cells*

Based on these results, we next sought to establish a FRET-based assay for detection of sortilin - p75$^{NTR}$ cross-linking in live cells. As sortilin and p75$^{NTR}$ physically interact through extracellular interactions [7], the intracellular domain of sortilin and p75$^{NTR}$ were substituted for the fluorescent proteins cerulean and venus and attached via a five amino acid polypeptide tether (**Figure 2A**). The constructs were then stably inserted into HEK293 cells, which do not express p75$^{NTR}$ and only very low amounts of endogenous sortilin. Western blots confirmed expression of both fusion constructs in HEK293 cells and fluorescent images showed expression of both proteins at the cell surface as well as a pool of newly synthesized or internalized proteins from the cell surface located in intracellular vesicles (**Figure 2B**, **2C**). We next ana-

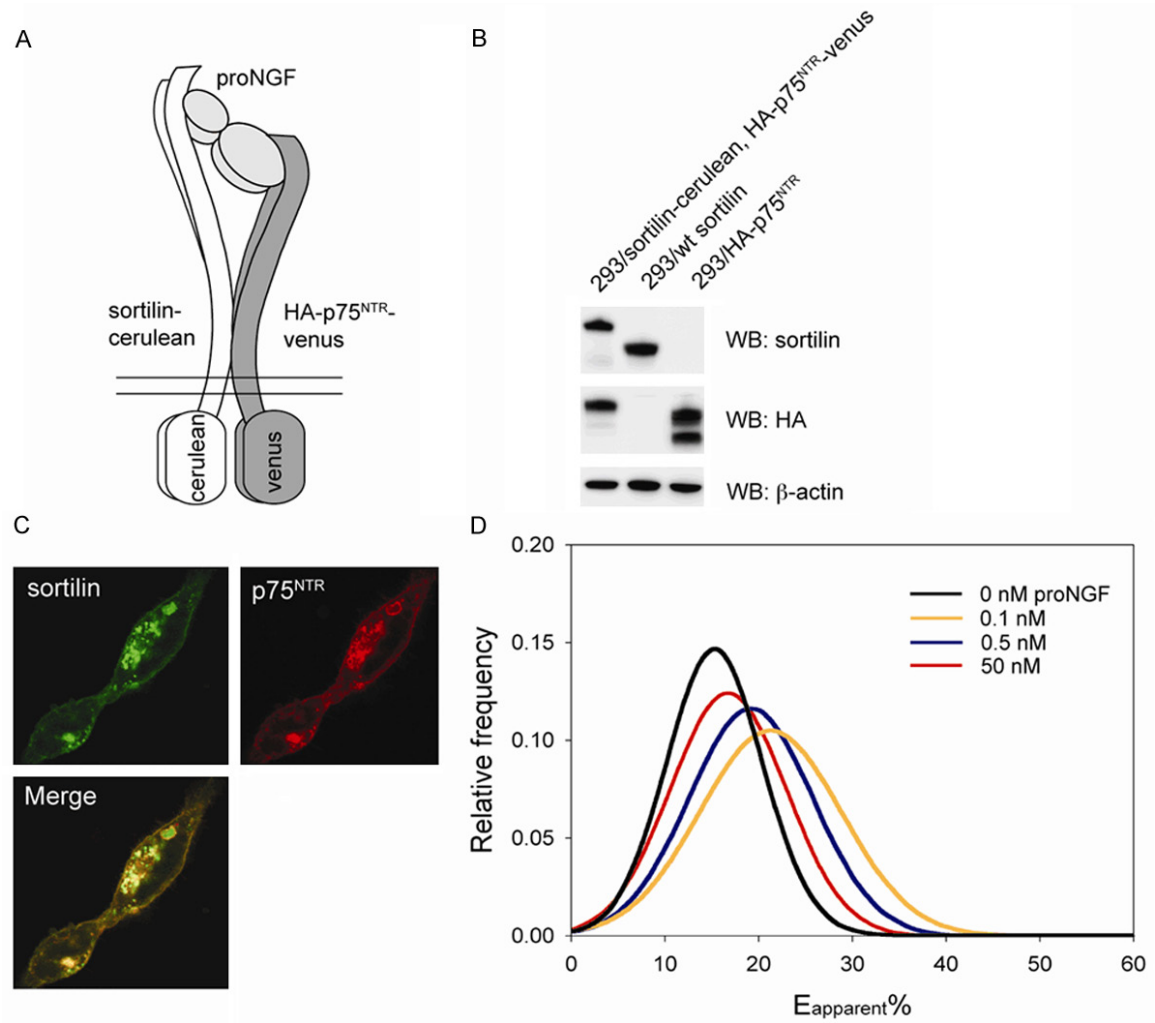


**Figure 2.** Establishing a FRET-based assay using sortilin-cerulean and p75$^{NTR}$-venus constructs in HEK293 cells. (A) Sortilin-cerulean and p75$^{NTR}$-venus constructs were generated by exchanging the sequence of the cytoplasmatic domain of the receptors for the sequence of the fluorescent proteins leaving the binding site for proNGF intact at the extracellular domain. (B and C) Co-expression of fusion proteins in stably transfected cells as revealed by western blotting (B) and confocal microscopy (C). (D) Addition of 0.1 or 0.5 nM proNGF increased FRET between sortilin-cerulean and p75$^{NTR}$-venus compared to untreated cells. Most groups were significantly different from untreated cells ($p < 0.001$), however no significant difference could be detected between untreated cells and the cells treated with 50 nM proNGF (n=10-14). All data are presented as the mean ± SD.

lyzed the effect of proNGF on sortilin - p75$^{NTR}$ cross-linking in these cells by use of FRET using confocal microscopy. 48 h post-seeding on coverslips, medium was exchanged to PBS containing proNGF and incubated 45 min before imaging the cells. In order to avoid any FRET signal stemming from receptors within vesicles, which may not be accessible to proNGF, only signals originating from the membrane surface was chosen for analysis (see materials and methods). By adding 0.1 or 0.5 nM proNGF, $E_{app}$% was significantly increased ~40% and ~18% compared to untreated cells (**Figure 2D**). However, addition of 50 nM proNGF had no significant effect. Thus, our experimental set-up using exogenously expressed fusion proteins in live HEK293 cells allowed detection of sortilin - p75$^{NTR}$ cross-linking induced by physiologically relevant concentrations of proNGF.

*Setting up a cellular FRET-based plate reader assay to follow sortilin - p75$^{NTR}$ interactions*

These results further encouraged us to establish a high throughput FRET-based assay using the stably transfected HEK293 cell line co-

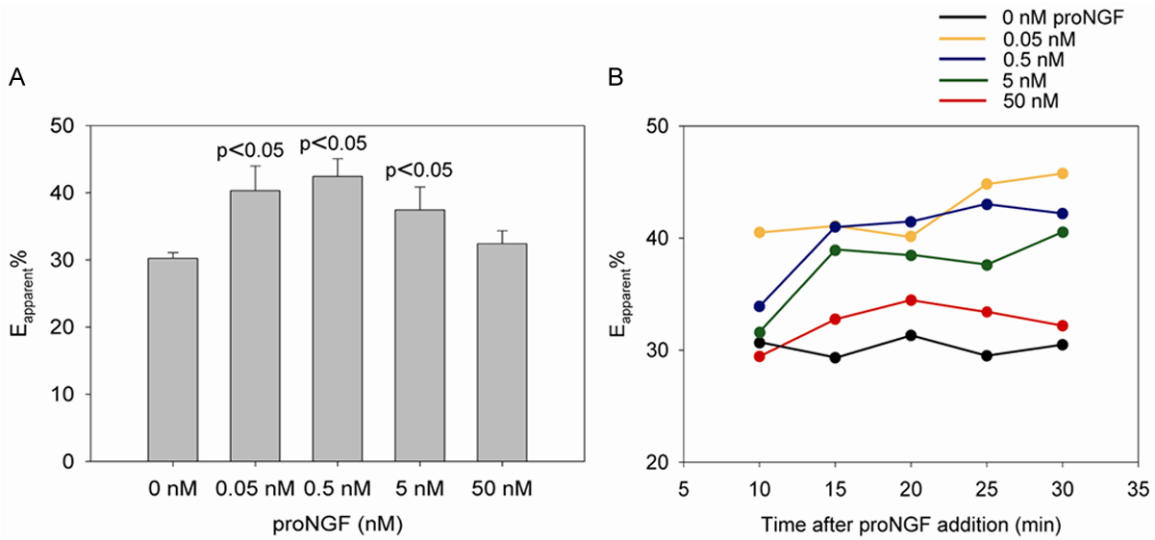


**Figure 3.** The effect of proNGF on the interaction between sortilin-cerulean and p75$^{NTR}$-venus using a standard fluorescent plate reader. A. Addition of 0.05, 0.5, or 5 nM proNGF significantly increased the $E_{app}$% by decreasing the distance between the receptors except for cells treated with 50 nM proNGF (n=3). B. During 30 min exposure to low amounts of proNGF, $E_{app}$% increased whereas the FRET-value remained fixed in the absence or in the presence of 50 nM ligand.

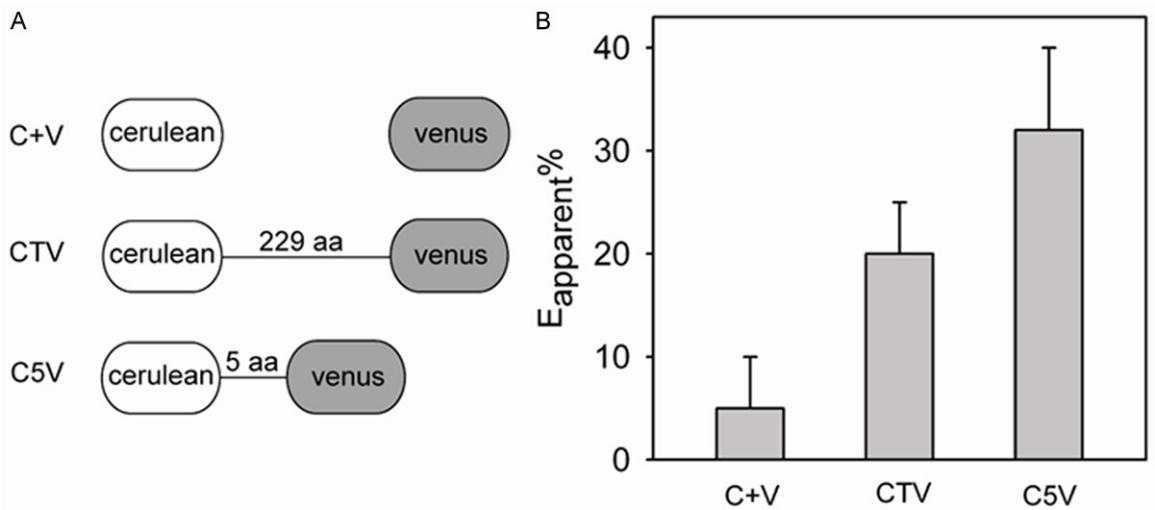


**Figure 4.** Decreasing the distance between cerulean and venus increases FRET in the plate reader assay. A. Schematic representation of used standard constructs. B. FRET efficiencies obtained using HEK293 cells transiently transfected with CTV, C5V, or co-transfected with plasmids encoding cerulean or venus (C+V) (n=3). All data are presented as the mean ± SD. aa, amino acids.

expressing sortilin-cerulean and p75$^{NTR}$-venus. Cells were seeded into 96-wells plates two days prior to the experiment. Medium was then carefully exchanged with PBS and proNGF added directly to wells and incubated for 30 min. Measurements were done at 20°C using a Victor3, 1420 Multilabel counter fluorescent plate reader from Perkin Elmer. Again, low amounts of proNGF i.e. 0.05, 0.5, 5 nM increased the interaction between sortilin and p75$^{NTR}$ significantly as $E_{app}$% was increased ~34%, ~43%, and ~25% compared to untreated cells. In contrast, high amounts of proNGF i.e. 50 nM had no significant effect (**Figure 3A**).

30 min time course experiments further showed that the FRET values remained constant for untreated cells whereas $E_{app}$% increased for

the proNGF-treated cells except for cells treated with 50 nM proNGF (**Figure 3B**).

To further characterize and validate our experimental set-up, we used a pair of FRET standards consisting of covalently fused fluorescent proteins cerulean and venus separated by an amino acid linker of variable length [17, 18]. For the standard C5V, a linker of five amino acids separates the fluorophores whereas the other standard CTV, contains a linker of 229 amino acids (**Figure 4A**). As the fluorophores of C5V are in close proximity, high levels of FRET is expected as compared to CTV, which is expected to result in a low FRET index due to the greater distance separating the fluorophores. Non-attached cerulean and venus (C+V) are expected to result in even lower FRET. Following transient transfection of HEK293 cells and using the FRET-based plate reader set-up, we calculated a FRET efficiency value of 5%, 20%, and 32% for cells expressing C+V, CTV and C5V (**Figure 4B**). Although the $E_{app}$% is somewhat lower than that published by Koushik et al. [17], the result shows the robustness of our assay by being able to detect the expected increase in FRET efficiency as the fluorophores are being brought together.

## Discussion

We have used a fluorescent plate reader combined with a FRET-based method to monitor the effect of proNGF on cross-linking of sortilin and p75$^{NTR}$ in live HEK293 cells with a stable expression of the fusion proteins sortilin-cerulean and p75$^{NTR}$-venus. The assay was tested using a pair of FRET standard constructs cerulean and venus separated by a linker of variable length. The results obtained with the standard constructs suggest that the differences in FRET induced by low levels of proNGF indeed reflect changes in the proximity of sortilin-cerulean and p75$^{NTR}$-venus. Importantly, the effective concentrations of proNGF used in our FRET-based plate reader assay lies within a physiologically relevant range i.e. 0.05-0.5 nM. This finding also applies to experiments using the confocal microscope for measurements. Interestingly, for all three experimental set-ups, cells treated with higher concentration of proNGF (~5-50 nM) had either no effect or even decreased the interaction rather than increasing it. This might be due to a competitive effect of proNGF, where both sortilin and p75$^{NTR}$ obtain ligand saturation and therefore each interact with a separate molecule of proNGF rather than sharing the ligand, thereby driving the two receptors apart. However, our results highlight the need to determine the optimal concentration of proNGF in the process of establishing the plate reader assay in order to promote maximal cross-linking of sortilin and p75$^{NTR}$.

FRET analysis was performed using the PFRET algorithm [19], which can be applied to regions of interest with sizes defined by the user, in principle down to the single-pixel level. It should be noted however, that the results acquired from the plate reader is calculated as an average over a population of cells within a well while the results presented from the microscopy measurements represents averages over 5x5 pixel.

The advantage of using the fluorescent plate reader for FRET measurements is that it easily and quickly delivers results and therefore is very useful for high troughput. Furthermore, a plate reader is typically available in most standard biochemical laboratories and will therefore not require investment in new expensive hardware.

It should be noted that the use of cells expressing sortilin and p75$^{NTR}$ fused to cerulean and venus results in a pool of intracellular located proteins, which are most likely not partaking in the interaction with proNGF. As the intracellular located proteins add to the fluorescent signal detected by the plate reader, the effect of proNGF could therefore be expected to be underestimated. It is therefore important to test the plate reader result on a confocal microscope where the intracellular contribution to the fluorescent signal subsequently can be subtracted. However, by using cells stably co-transfected with sortilin-cerulean and p75$^{NTR}$-venus, the very same cell line can be used for several different FRET-based techniques based on a plate reader, microscope, and flow cytometer.

In conclusion, this paper describes the establishment of a FRET-based fluorescent plate reader assay using live cells to monitor sortilin - p75$^{NTR}$ cross-linking and the influence of proNGF on this interaction. The experimental set-up may provide a useful tool for identifying compounds interfering with cross-linking of

sortilin - p75$^{NTR}$. Using this set-up, we have now initiated work aimed at identifying compounds inhibiting sortilin - p75$^{NTR}$ cross-linking induced by proNGF.

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## Disclosure of conflict of interest

None.

## Abbreviations

ASBT, acceptor spectral bleed trough; DSBT, donor spectral bleed through; $E_{app}$%, apparent FRET efficiency; FCS, fetal calf serum; FRET, fluorescence resonance energy transfer; HEK-293 cells, Human embryonic kidney 293 cells; NGF, nerve growth factor; p75NTR, p75 neurotrophin receptor; RN22 cells, rat schwannoma cells; ROI, region of interest.

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