1 **Characterization of clostridium botulinum neurotoxin serotype A (BoNT/A) and fibroblast**

2 **growth factor receptor interactions using a novel receptor dimerization assay**

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4 Short title: BoNT/A and FGFR interactions

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ABSTRACT

26   Clostridium botulinum neurotoxin serotype A (BoNT/A) is a potent neurotoxin that also serves as

27   an effective therapeutic for a variety of neuromuscular and glandular diseases and disorders. The

28   observed pharmacological effect of BoNT/A is due to specific targeting and entry into motor

29   nerve terminals within muscles followed by cleavage of the SNARE protein, SNAP-25, inducing a

30   block in neurotransmission and temporary muscular paralysis. Specific binding and

31   internalization of BoNT/A into neuronal cells are mediated by its binding domain (H_C/A), which

32   binds to GT1b ganglioside and protein cell surface receptors. Previously, fibroblast growth factor

33   receptor 3 (FGFR3) was identified as a BoNT/A receptor, in addition to synaptic vesicle protein

34   (SV2). To further study BoNT/A interactions with FGFRs, an FCS & TIRF receptor dimerization

35   assay was developed to measure dimerization of FGFRs in live cells, as FGFR dimerization can be

36   considered an indirect measure for receptor-ligand binding interaction and downstream

37   signaling. The ability of H_C/A to facilitate dimerization of three FGFR subtypes (FGFR1-3) was

38   assessed. Recombinant H_C/A (rH_C/A) was shown to dimerize FGFR subtypes in the rank order

39   FGFR3c > FGFR2b > FGFR1c. With potencies (EC_{50} values) defined as the concentration of ligand

40   required to dimerize 50% of the receptors, wild type rH_C/A dimerized FGFR3c with an EC_{50} of 24

41   nM, similar to FGF9, a native FGFR3c ligand, which had an EC_{50} of 15 nM, while FGFR1c and

42   FGFR2b required higher rH_C/A concentrations (≥100 nM and 68 nM, respectively). Furthermore,

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43   addition of the GT1b ganglioside to the culture media resulted in increased dimerization, whereas

44   a ganglioside mutant variant of H_C/A (rH_C/A W1266L;Y1267S) showed decreased dimerization.

45   Interestingly, reduced dimerization was also observed with an SV2 mutant variant of H_C/A (rH_C/A

46   T1145A;T1146A). These results support a model wherein BoNT/A interacts with FGFRs,

47   gangliosides, and SV2 on the cell surface to facilitate cell uptake.

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INTRODUCTION

Botulinum Neurotoxin Type A (BoNT/A) is a 150 kDa metalloenzyme belonging to the family of neurotoxins produced by *Clostridium botulinum* which causes temporary muscle paralysis by inhibiting acetylcholine release at the neuromuscular junction [1-4]. The neuronal specificity and high potency of BoNT/A has allowed its use in the treatment of a large number of medical and aesthetic conditions [3, 5-7], relying on injection of picomolar (pM) concentrations of the toxin. Though BoNT/A has been the subject of extensive study, greater understanding of the complex mechanism associated with BoNT/A's neuronal specificity and cellular entry could lead to further therapeutic applications.

BoNT/A is a single-chain protein activated by proteolytic cleavage to form a 150 kDa di-chain molecule. The di-chain is comprised of a light chain (L_C/A) which encodes a Zn^{2+} dependent endopeptidase (~50 kDa), linked by a single disulfide bond and non-covalent interactions to a ~100 kDa heavy chain (HC) containing the receptor binding and translocation domains [8]. The 50 kDa receptor binding domain, H_C/A, is located at the C-terminal half of the HC and mediates specific binding and internalization of the toxin into neurons. Following internalization, the translocation domain (H_N) of BoNT/A residing at the N-terminal half of the HC facilitates the translocation of L_C/A from the endocytic vesicle into the cytosol. Once in the cytosol, L_C/A enzymatically cleaves the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), synaptosomal-associated protein 25 (SNAP-25) [9, 10], which is essential for mediating vesicular fusion and exocytosis. Cleavage of SNAP-25 leads to inhibition of neurotransmitter/neuropeptide release, including acetylcholine, from neuronal cells and is

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82   responsible for BoNT/A's observed pharmacological effects on smooth and skeletal muscles and

83   glands [1, 11, 12].

84   Initially, BoNT/A binds with relatively low affinity (K_D ~200 nM) to gangliosides, including

85   GT1b, which are abundant at the presynaptic membrane and serve to trap and concentrate

86   BoNT/A within the extracellular matrix [13-16]. H_C/A mutations (W1266L;Y1267S)[16] have been

87   identified which disrupt binding to GT1b and likely additional gangliosides (e.g., GD1a, GD1b,

88   GQ1b, and GM1), which bind to BoNT/A with lower affinity compared to GT1b [14-17]. Once

89   anchored close to the membrane, BoNT/A interacts with one or more relative high affinity

90   protein receptors, including the synaptic vesicle protein, SV2 (K_D ~100 nM) [18-23], and FGFR3

91   (K_D ~15 nM) [24, 25]. Mutations within H_C/A that disrupt binding to SV2 have been identified,

92   including T1145A;T1146A [20], and G1292R [19]. The observed in vivo selectivity of BoNT/A for

93   specific classes of neuronal cells is likely due to avidity upon binding to multiple receptors, which

94   may also serve as a requirement to trigger internalization into the neuronal cell via endocytosis

95   [13-16]. Interaction of BoNT/A with multiple receptors could provide an evolutionary advantage

96   for the toxin, since it decreases the likelihood of host specific mutations resulting in toxin

97   resistance. A similar strategy is known from other pathogens, including *Herpes simplex virus*

98   (HSV) [26], *Trypanosoma cruzi* (Chaga's disease) [27, 28], and human immunodeficiency virus

99   (HIV) [29, 30]. Influenza A virus infection has also been suggested to involve FGFRs [31]. Previous

100   protein complex immunoprecipitation results demonstrated an interaction between FGFRs and

101   SV2 in neuronal cells [24], further suggesting the possibility of a multi-receptor BoNT/A complex.

102   FGFR3 is one of four receptor-tyrosine kinases (FGFR1–4) that act as receptors for FGFs.

103   FGFR1–3, but not FGFR4, exist in three different splice variants, a–c, which differ in their

104   extracellular ligand binding domains, each with differing ligand binding affinities and specificities

105   [32, 33]. The a isoform variants terminate early, resulting in a secreted extracellular FGF-binding

106   protein [34-37]. The b isoform variants, including FGFR2b, are primarily expressed in tissues of

107   epithelial (surface tissue) origin, while the c isoform variants, including FGFR1c and FGFR3c, are

108   primarily expressed in tissues of mesenchymal (connective tissue) origin. Native ligands for FGFRs

109   are generally produced by either epithelial or mesenchymal cells and act on opposite tissue type

110   FGFRs. An exception is FGF1, which binds to both b and c FGFR isoforms [38, 39]. There are 22

111   known FGF ligands that bind with different affinity and selectivity to the different FGFR splice

112   variants. For example, FGF4 binds to FGFR1c > 2c > 3c, while FGF9 binds to FGFR3c > 2c > 1c and

113   3b, and FGF10 binds to FGFR2b > 1b [35, 37, 39, 40]. Most FGFs interact locally with FGFRs in a

114   paracrine or autocrine manner, although a number of FGFs, including FGF19, FGF21, and FGF23,

115   act like hormones in an endocrine manner [36, 41]. Selectivity and affinity in vivo is achieved via

116   interactions with co-receptors, including: heparin, heparan sulfate (HS), neural cell adhesion

117   molecule (NCAM), cadherin, integrin, Klotho, anosmin, neuropilin, and fibronectin, which

118   interact with both FGFs and FGFRs [42, 43]. Gangliosides have also been reported to be co-

119   receptors for FGFRs, affecting ligand binding, receptor dimerization, receptor activity, and

120   subcellular localization [44, 45].

121   This tissue specific expression of the ligands, receptors, and co-receptors, guides tissue

122   and organ growth and development. FGFR signaling upon ligand binding and receptor activation

123   is associated with numerous cellular functions, including development, homeostasis, and

124   metabolism. FGFRs are activated by dimerization induced by co-receptor and ligand binding,

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125   which enables the cytoplasmic kinase domains to transphosphorylate one another at specific

126   tyrosine residues [34-37, 46].

127   As noted above, the binding domain of botulinum neurotoxin, rH_C/A, has been shown to

128   bind FGFR in vitro and in cells [47, 48]. To further study wild-type (wt) and mutant variants of

129   rH_C/A interactions with FGFRs and compare these to native FGF ligand interactions, a novel

130   receptor dimerization cell-based assay was developed. Total internal reflection fluorescence

131   (TIRF) microscopy was combined with fluorescence correlation spectroscopy (FCS) and number

132   and brightness (N&B) analysis to develop an assay, hereafter referred to as the "FCS & TIRF

133   receptor dimerization assay", that measures receptor dimerization in live cells. By combining the

134   two methods, the FCS & TIRF receptor dimerization assay allows monitoring of receptor

135   dimerization at the cell membrane in real-time; in the presence of co-receptors, in any cell type,

136   without the need to create a custom reporter cell line or reliance on the use of functional

137   reporters, e.g. receptor phosphorylation sites or down-stream kinases.

138   In the study presented here, the FCS & TIRF receptor dimerization assay was used to

139   evaluate FGFR dimerization in the presence of wt and mutant variants of rH_C/A. FGFR

140   dimerization with native FGFs possessing known receptor selectivity was evaluated in parallel. In

141   addition, the impact of ganglioside and SV2 receptor interactions on H_C/A mediated FGFR

142   dimerization was studied. To better understand the specificity of the interaction between rH_C/A

143   and FGFRs, binding to another tyrosine kinase growth factor receptor, epidermal growth factor

144   receptor (EGFR), was also evaluated. Our results demonstrate that rH_C/A, in the presence of

145   gangliosides and SV2, can induce dimerization of FGFRs with a preference towards FGFR3c,

7

146   further supporting a functional role of FGFRs in BoNT/A cellular binding, as previously suggested

147   [24].

148

MATERIALS and METHODS

150   **Materials.** All cell culture medium and transfection reagents were from ThermoFisher (Carlsbad,

151   CA) unless otherwise stated. FGF proteins [2, 4, 13, 14] and EGF were from R&D Systems

152   (Minneapolis, MN). Collagen IV coated glass bottom 35 mm imaging dishes were from MatTek

153   (Ashland, MA). Rat pheochromocytoma cells (PC-12) and fetal bovine serum (FBS) were from

154   ATCC (Manassas, VA). GT1b was from Enzo Life Sciences (Farmingdale, NY). C-HaloTag-FGFRs,

155   which included FGFR1c (EX-Y2820-M50, accession number: NM_023110.2, Homo sapiens

156   fibroblast growth factor receptor 1), FGFR2b (EX-Z6888-M50, accession number:

157   NM_001144913.1, Homo sapiens fibroblast growth factor receptor 2), and FGFR3c (EX-M0098-

158   M50, accession number: NM_000142.4, Homo sapiens fibroblast growth factor receptor 3), and

159   C-HaloTag-EGFR (EX-A8661-M50, accession number: NM_005228.4, Homo sapiens epidermal

160   growth factor receptor) constructs were from Genecopoeia (Rockville, MD). AlexaFluor488 Halo

161   ligand (AF-488) was from Promega (Madison, WI). Protease inhibitor cocktail was from Sigma-

162   Aldrich (St. Louis, MO). Benzonase nuclease and rLysozyme were from EMD Sigma (Billerica, MA).

163   HisTRAP FF and DEAE columns were from GE Healthcare (Chicago, IL).

164

165   **Cell culture.** PC-12 cells were cultured on 100 mm collagen IV plates (cat. no. 354453) using

166   complete growth media consisting of RPMI media with 2 mM GlutaMAX™, 10% FBS, 10 mM

167   HEPES, 1 mM Sodium Pyruvate, 100 U/ml Penicillin, and 100 µg/ml Streptomycin. Cells were

168   seeded onto imaging dishes and incubated in complete medium supplemented with 25 µg/mL

169   GT1b (unless otherwise noted) until reaching confluency (~4 days). The cells were maintained at

170   37°C with 5% CO_2.

171

172   **Transfection of cells.** PC-12 cells were plated at ~$1x10^6$ cells per 35 mm collagen IV coated glass

173   bottom imaging dish and transfected with a mammalian expression plasmid containing the DNA

174   sequence encoding C-HaloTag-FGFR1c, C-HaloTag-FGFR2b, C-HaloTag-FGFR3c, or C-HaloTag-

175   EGFR. A DNA solution was prepared by adding and mixing (by inversion) 2.5 µg of plasmid with

176   100 µL OPTI-MEM media. The transfection solution was prepared by adding and mixing (by

177   inversion) 5.0 µL of Lipofectamine 2000 with 100 µL of OPTI-MEM media. Once the DNA and

178   transfection solutions were mixed by inversion and incubated at room temperature for 30

179   minutes, the 200 µL aliquot of the lipofectamine/plasmid solution was added to the plate

180   containing the cells. After 6 hours of incubation, the medium was replaced with complete

181   medium with or without GT1b and incubated for an additional 24 hours prior to addition of the

182   HaloTag ligand.

183

184   **Treatment of cells with Halotag ligand and test compound for cell imaging**. After transfection,

185   the cells were incubated with 500 nM AlexaFluor488 Halo ligand for 15 hours. The cells were

186   then washed three times (10 minutes/wash) with fresh complete medium to remove free

187   ligand and incubated in serum-free medium (complete medium minus FBS, plus B-27 and N-2)

188   for 3-4 hours prior to imaging.

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189

190 **Expression and purification of wildtype and mutant variants of H$_C$/A.** Recombinant H$_C$/A (rH$_C$/A;

191 spanning aa 876-1296 of full length BoNT/A1; GenBank accession no. AF48874) was cloned into

192 pET28a+ (N-terminal His6-tag). The DNA was codon optimized for expression in *Escherichia Coli*.

193 The variants of H$_C$/A, (W1266L;Y1267S [16]) and H$_C$/A (T1145A;T1146A [20]) were made by site-

194 directed mutagenesis (Genewiz, NJ). The variants were expressed and purified as previously

195 described [24]. Briefly, chemically competent BL21(DE3) *E. coli* (Thermo Fisher Scientific,

196 Waltham, MA) were transformed with a rH$_C$/A expression plasmid. Cells were grown at 37°C until

197 OD600 reached 0.7 and expression induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside

198 (IPTG). After 16 hours of growth at 22°C, cells were harvested via centrifugation and lysed in buffer

199 containing: 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, 10 mM DTT, 5% (v/v) glycerol,

200 cOmplete™ EDTA-Free Protease Inhibitor Cocktail (MilliporeSigma, St. Louis, MO), 150 mU/mL

201 rLysozyme, and 50 mU/mL benzonase nuclease, and then sonicated for 5 minutes. Lysate was

202 cleared through high speed centrifugation and subjected to Immobilized Metal Ion Affinity

203 Chromatography (IMAC) (HisTrap™), buffer exchange (desalting column) and Ion Exchange

204 Chromatography (IEX) using the ÄKTAxpress system (GE Healthcare Bio-Sciences, Pittsburgh, PA).

205 Protein (50 mM HEPES, pH 7.4, 150 mM NaCl) was quantified using NanoDrop™ 2000

206 spectrophotometer (Thermo Fisher Scientific).

207

208 **Image acquisition.** Images were recorded on a Nikon Eclipse Ti TIRF microscope using a 60X 1.45

209 NA TIRF oil objective. Approximately 1000 frames were collected per cell and captured on a

210 cascade 512B EMCCD camera (Photometrics, Tucson, Az) at 100 frames per second using software

10

211   written in-house [48, 49]. AF-488 was excited at 488 nm (Sapphire SF 488; Coherent, Santa Clara,

212   CA) with less than 30% laser power using an excitation filter cube (488/10 nm bandpass and 500

213   nm long pass emission filter; Chroma, Bellows Falls, VT) within the infinity space. Intensity images

214   were recorded for each transfected HaloTag receptor in the absence of FGF or rH$_C$/A protein ligand

215   at both the beginning and end of each experiment from two separate dishes. The cells were then

216   treated for 30 minutes with increasing concentration of ligand, reconstituted in PBS containing

217   0.1% Bovine Serum Albumin at a final concentration of 0.1 to 200 nM, prior to recording TIRF

218   intensity images. Dark counts for the EMCCD camera were recorded at the beginning and end of

219   each experiment. Free AF-488 (90 nM) in serum-free medium was used as the monomeric

220   standard. Images were processed using the N&B function on the software platform SimFCS. Using

221   FCS, the brightness of a particle as well as the number of particles in a given volume (Number and

222   Brightness Analysis, S1) were separately obtained to determine the degree of aggregation of

223   proteins in solution [47-53].

224   The average normalized brightness value ± standard deviation (SD) for each concentration

225   of ligand was calculated based on at least 20 individual cells on 4 independent days. The results

226   were plotted using GraphPad 7.02 Prism and fitted to a two- or three-parameter logistic curve

227   (2PL or 3PL) using the log of the concentration to estimate the EC50 values and the 95%

228   confidence intervals (95% CI). For the fitting, the curves were constrained with bottom >1 (3PL)

229   or =1 (2PL) and the top =2, reflecting a transition from monomer to dimer state. For graphical

230   presentation, the concentrations are plotted on a log scale.

231

RESULTS

233   **FCS & TIRF receptor dimerization assay for studying receptor dimerization in live cells.**

234

235   In the TIRF (Total Internal Reflection Fluorescence) method, the optics of the instrument

236   are adjusted such that the exciting light will be reflected from the interface, i.e., the glass surface

237   supporting the cell. However, some of the energy of the incident beam will penetrate through

238   the interface, creating what is termed an evanescent field which extends a very short distance

239   on the order of 100 nm into the cell. Hence, this evanescent beam will only be able to excite

240   fluorophores which are located near the cell surface—i.e., the plasma membrane of the target

241   cell (**Figure 1**) [54]. Fluorescence fluctuation spectroscopy and number and brightness (N&B)

242   analysis is an emerging method for analysis of molecular interactions in living cells and is based

243   on the statistical analysis of signal fluctuations emitted by fluorescently labeled molecules [47-

244   57] (**S1 Figure**). The method utilizes the fact that fluorescently labeled proteins produce intensity

245   fluctuations as they pass through a small observation volume. Since the method relies on

246   movement of fluorescent molecules, only mobile molecules are evaluated. While the average

247   intensity in two sample volumes containing the same number of fluorophores may be the same,

248   the fluctuations in intensity depend on the molecular brightness of the fluorescent protein

249   molecules in the samples, and the magnitude of the fluctuations contains information about

250   molecule concentration and the oligomeric state. By measuring the average fluorescence

251   intensity within each pixel (photon counts per second per molecule), the average oligomeric state

252   within each pixel is determined by calculating the molecular brightness, which is directly related

253   to the stoichiometry of fluorophores in a protein complex, and normalizing it to a molecular

254   brightness monomer standard. For example, if a fluorescently labeled monomeric FGFR is found

255   to homodimerize under addition of a ligand, a 2x increase in the average molecular brightness

256   would be observed compared to the monomeric brightness standard, and the number of pixels

257   with 2x normalized average molecular brightness would increase (**Figure 2B; S2 Figure**).

258

259   **Fig 1. Illustration of the total internal reflection fluorescence (TIRF) method.**

260

261   **Fig 2. Depiction of intensity and molecular brightness images. A.** (Intensity) Typical single cell

262   intensity images are shown for cells that have not been exposed to FGF9 ligand (left column) and

263   cells exposed to 25 nM FGF9 ligand (right column). **B.** (Molecular brightness) The normalized

264   average molecular brightness images for each cell indicate monomer (red) and dimer (green)

265   numbers and distribution throughout the cell with corresponding pixel counts, which is the

266   number of pixels that contain dimer or monomer, respectively.

267

268   Using the FCS & TIRF receptor dimerization assay, the ratio of monomer to dimer FGFRs

269   on the plasma membrane of neuronal PC-12 cells was measured, with and without addition of

270   FGF ligand or rH_C/A protein. We focused on FGFR1c, FGFR2b, and FGFR3c, which represents

271   mesenchymal, epithelial, and neuronal FGFRs, respectively [32, 58, 59]. Briefly, a rat

272   neuroendocrine tumor cell line, PC-12, was transiently transfected with FGFRs containing a C-

273   terminal HaloTag, which is a modified haloalkane dehalogenase designed to covalently bind to

274   exogenously added synthetic ligands bound to a fluorophore (HaloTag ligands; AF488 [cell

275   impermeable for sell surface labeling]) [55] (FGFR3c-AF488). A dose dependent increase in

276   ERK1/2 phosphorylation was observed in transfected versus non-transfected cells upon addition

277 of native ligands, FGF2 and FGF9, at 2, 9, and 21 nM, indicating that the C-terminal HaloTag does

278 not disrupt FGF receptor function (data not shown). TIRF intensity measurements of transfected

279 PC-12 cells indicated a non-homogenous distribution of fluorescence, both with and without the

280 ligand (**Figure 2A**), perhaps indicative of aggregated receptor proteins. Immobile fluorophores do

281 not fluctuate, so any potentially aggregated protein will not be analyzed. Calculation of the

282 average molecular brightness, in the absence of ligand, showed that the values for FGFR3c-AF488

283 were higher (1.1-1.2-fold) than the Alexa488 fluorophore molecular brightness monomer

284 standard, suggesting that 10-20%of the receptors are in preformed dimers. Dimerization in the

285 absence of ligand has previously been reported for EGFR under overexpression conditions that

286 favor high receptor densities on the plasma membrane [60]. Data was collected from cells with

287 an emission of 10,000-15,000 counts per cell, corresponding to approximately 100,000-150,000

288 receptors per cell, based on the Alexa488 fluorophore monomer standard. This criterion was

289 used to ensure that the level of receptor expression was relatively constant across measured

290 cells, avoiding cells with very low or high receptor densities. Typical intensity images

291 corresponding to monomer and dimer populations are shown in **Figure 2**, where saturation with

292 native ligand (FGF9 for FGFR3c) caused ~2x increase in normalized average molecular brightness,

293 consistent with transition to a 100% dimerized state.

294

295 **rH$_C$/A induces FGFR3c dimerization.**

296

297   Previously, rH$_C$/A was shown to bind to extracellular loops 2 and 3 of FGFR3 in vitro and

298 on cells with a KD of ~15 nM, which is similar to the affinity of native FGFR ligands, fibroblast

14

299   growth factors (FGFs) [20]. To further validate the FCS & TIRF receptor dimerization assay and to

300   compare the ability of FGFs and rH$_C$/A to dimerize FGFR3c, PC-12 cells transfected with FGFR3c-

301   AF488 were treated with rH$_C$/A. In parallel, two native FGFR ligands were analyzed; FGF9, a

302   known agonist ligand for FGFR3c and FGF10, a known agonist ligand for FGFR2b but not FGFR3c.

303   As expected, addition of FGF9 resulted in a dose-dependent increase in average normalized

304   molecular brightness, whereas treatment with FGF10 did not (**Figure 3**). EC$_{50}$ values for FGFR3c

305   dimerization, defined as concentration of ligand resulting in 50% receptor dimerization, were 18

306   nM (95% CI [12, 24]) for FGF9 and >200 nM for FGF10 (**Table 1**). Notably, the ability of rH$_C$/A to

307   dimerize FGFR3c was similar to FGF9 with an EC$_{50}$ of 27 nM (95% CI; 18, 41), suggesting that rH$_C$/A

308   has FGFR3c agonist-like properties (**Figure 3; Table 1**).

309

310   **Fig 3. rH$_C$/A induces FGFR3c dimerization.** Native ligand FGF9 (black) (EC$_{50}$ = 18 nM [95% CI; 12,

311   24]) and BoNT/A binding domain (rH$_C$/A) (green) (EC$_{50}$ = 27 nM [95% CI; 18, 41]), but not FGF10

312   (red) (EC$_{50}$ >100 nM), dimerize FGFR3c expressed on neuronal PC-12 cells. Points represent the

313   average normalized brightness values ±SD from greater than 30 cells collected on 4

314   independent days.

315

316   **Table 1. FCS&TIRF receptor dimerization assay – Result summary**

Receptor	Ligand	GT1b	Function	EC$_{50}$	95% CI*
FGFR1c	FGF4	Yes	Native FGFR1c Ligand	17 nM	(8, 35)
	FGF10		Native FGFR2b Ligand	>100 nM	N/A
	rH$_C$/A		BoNT/A binding domain	163 nM	(123, 203)**

15

FGFR2b	FGF10		Native FGFR2b Ligand	19 nM	(13, 26)
	FGF4		Native FGFR1c Ligand	>100 nM	N/A
	rH_C/A		BoNT/A binding domain	70 nM	(59, 82)
FGFR3c	FGF9		Native FGFR3c Ligand	18 nM	(12, 24)
	FGF10		Native FGFR2b Ligand	>200 nM	N/A
	rH_C/A		BoNT/A binding domain	27 nM	(18, 41)
	rH_C/A	No	BoNT/A binding domain	44 nM	(36, 54)
	rH_C/A W1266L;Y1267S	Yes	BoNT/A binding domain, ganglioside mutant	143 nM	(127, 161)
	rH_C/A T1145A;T1146A		BoNT/A binding domain, SV2 mutant	78 nM	(53, 115)
EGFR	EGF		Native EGFR Ligand	14 nM	(11, 19)
	rH_C/A		BoNT/A binding domain	>150 nM	N/A

317   ***Based on 3PL with bottom >1, top =2**

318   ****Based on 2PL with bottom = 1, top = 2**

319

320   **rH_C/A induced dimerization of FGFR3c is dependent upon GT1b ganglioside.**

321

322   Gangliosides, such as GT1b, serve as abundant, low affinity receptors for BoNT/A at the

323   neuromuscular junction [13-16]. Since gangliosides can also function as co-receptors for FGFRs

324   [44, 45], the FCS & TIRF receptor dimerization assay was performed with and without addition of

325   exogenous GT1b to directly assess its potential role in rH_C/A induced FGFR3c dimerization. A

326   ganglioside mutant variant of H_C/A (rH_C/A W1266L;Y1267S [16]) was included to assess potential

327   interactions with endogenous ganglioside produced in culture. Previously, it was shown that

328   rH_C/A W1266L;Y1267S protein lost its ability to bind to neuronal cells but maintained its ability

329   to bind and cross epithelial barriers, and that antibodies raised against rH_C/A W1266L;Y1267S

330   protected against full length BoNT/A at the neuromuscular junction [16], suggesting that the

331   protein was folded and stable. Consistent with this, we observed that the mutant rH_C/A

332   W1266L;Y1267S protein expresses well and is soluble, similar to the wt rH_C/A protein (data not

333   shown). In the absence of exogenous GT1b, rH_C/A showed reduced potency (EC_{50} = 44 nM [95%

334   CI; 36, 54] vs EC_{50} = 27 nM [95% CI; 18, 41]) (overlapping 95% CI) and was less effective at

335   dimerizing FGFR3c than when GT1b had been supplemented, failing to induce complete FGFR3c

336   dimerization even at 200 nM rH_C/A (**Figure 4; Table 1**). Cells treated with rH_C/A W1266L;Y1267S

337   in the presence of exogenous GT1b, showed further decreased potency (EC_{50} = 143 nM [95% CI;

338   127, 161]) (**Figure 4; Table 1**). Interestingly, dimerization of FGFR3c by rH_C/A in the absence of

339   exogenous GT1b was higher than dimerization by rH_C/A W1266L;Y1267S, suggesting that rH_C/A

340   dimerization of FGFR3c utilizes endogenic expressed gangliosides in PC-12 cells. These results

341   suggest that the presence of GT1b and likely additional gangliosides on the cell surface augments

342   rH_C/A binding and dimerization of the FGFR3 receptor.

343

344   **Fig 4. GT1b augments rH_C/A induced FGFR3c dimerization.** BoNT/A binding domain (rH_C/A) is

345   more potent at dimerizing FGFR3c in the presence of ganglioside (GT1b) (green vs black, EC_{50} =

346   27 nM [95% CI; 18, 41] vs EC_{50} = 44 nM [95% CI; 36, 54]), and a BoNT/A binding domain (rH_C/A)

347   ganglioside mutant variant (W1266L;Y1267S) has further reduced ability to dimerize FGFR3c

348   (red) (EC_{50} = 143 nM [95% CI; 127, 161]). Points represent the average normalized brightness

349   values ±SD from greater than 30 cells collected on 4 independent days.

350

351   **rH_C/A dimerizes FGFRs in the rank order FGFR3c > FGFR2b > FGFR1c.**

352

353   As the FGFR subtypes (FGFR1-4) share a high degree of structural and functional

354 homology, it was of interest to assess the affinity of rH_C/A to other FGFRs, including FGFR1c and

355 FGFR2b (**Figure 5; Table 1**). As expected, ligands known to bind specifically to these receptors

356 (i.e. FGF4/FGFR1c and FGF10/FGFR2b) caused an increase in molecular brightness with complete

357 dimerization appearing around 50-100 nM. The EC_{50} for FGF4 induced FGFR1c dimerization and

358 FGF10 induced FGFR2b dimerization were 17 nM (95% CI; 8, 35) and 19 nM (95% CI; 13, 26),

359 respectively. No increase in molecular brightness was observed with the non-binding receptor

360 ligand pairings FGF10/FGFR1c and FGF4/FGFR2b. Addition of rH_C/A resulted in a dose-dependent

361 increase in both FGFR1c and FGFR2b dimerization, but with lower potency compared to the

362 native ligands and compared to rH_C/A-induced FGFR3c dimerization. The EC_{50} for rH_C/A induced

363 FGFR1c and FGFR2b dimerization were 163 nM (95% CI; 123, 203) and 70 nM (95% CI; 59, 82),

364 respectively.

365

366 **Fig 5. FGFR binding preference of rH_C/A. A.** BoNT/A binding domain (rH_C/A) (green) dimerizes

367 FGFR1c with reduced potency compared to FGFR3c [58]. A known native FGFR1c ligand, FGF4

368 (black) (positive control), dimerizes FGFR1c (EC_{50} = 17 nM [95% CI; 8, 35]), while a known native

369 FGFR2b ligand, FGF10 (red), does not (EC_{50} >100 nM). Points represent the average normalized

370 brightness values ±SD from greater than 20 cells collected on 4 independent days. **B.** BoNT/A

371 binding domain (rH_C/A) (green) dimerizes FGFR2b with reduced potency compared to FGFR3c

372 (EC_{50} 70 nM [95% CI; 59, 82] vs 27 nM (95% CI; 18, 41]) (**Figure 3**). A known native FGFR2b ligand,

373 FGF10 (black) (positive control), dimerizes FGFR2b (EC_{50} = 19 nM [95% CI; 13, 26]), while a known

374   native FGFR1c ligand, FGF4 (red), does not (EC_{50} >100 nM). Points represent the average

375   normalized brightness values ±SD from greater than 30 cells collected on 4 independent days.

376   *Based on 2PL with bottom = 1, top = 2.

377

378   **rH_C/A induced dimerization of FGFR3c is dependent on SV2.**

379

380   BoNT/A's interaction with SV2 has been well characterized and mapped to the fourth

381   luminal domain of SV2 and a beta-strand within H_C/A [19, 20]. As previous results suggested that

382   FGFR and SV2 interact in cells [24], it was of interest to assess a potential role for SV2 binding

383   during H_C/A induced FGFR dimerization. Addition of an SV2 mutant variant of H_C/A (rH_C/A

384   T1145A;T1146A [20]) resulted in a dose-dependent increase in dimerization of FGFR3c, but with

385   reduced potency (EC_{50} = 78 nM [95% CI; 53, 115]) compared to the wild type variant (EC_{50} = 27

386   nM [95% CI; 18, 41]) (**Figure 6; Table 1**). Similar to the effect observed with GT1b, these data

387   suggest that residues in H_C/A which interact with SV2 also affect binding to FGFRs, either directly

388   or indirectly. It is unclear how the T1145A;T1146A mutation affects the folding of rH_C/A.

389   However, we have observed that the mutant rH_C/A T1145A;T1146A protein expresses well and

390   is soluble, similar to the wt rH_C/A protein (data not shown). Previously, it was shown that rH_C/A

391   T1145A;T1146A bound with lower affinity to a recombinant SV2C luminal domain in vitro [20].

392   The fact that it still binds, albeit with reduced affinity, also suggests that the protein is folded

393   similarly to the wt protein.

394

395 **Fig 6. SV2 augments rH$_C$/A induced FGFR3c dimerization.** A BoNT/A binding domain SV2

396 mutant variant (rH$_C$/A T1145A;T1146A) (black) has reduced ability to dimerize FGFR3c

397 compared to a wt BoNT/A binding domain (rH$_C$/A) (green), EC$_{50}$ = 78 nM (95% CI; 53, 115) vs 27

398 nM (95% CI; 18, 41). Points represent the average normalized brightness values ±SD from

399 greater than 30 cells collected on 4 independent days.

400

401 **rH$_C$/A does not dimerize EGFR.**

402

403 To assess the selectivity of rH$_C$/A for FGFRs versus similar growth factor receptors, the

404 ability of rH$_C$/A to dimerize EGFR was assessed. EGFR is another tyrosine kinase growth factor

405 receptor that shares structural and functional homology with FGFRs. The native ligand is EGF [61-

406 63]. As expected, addition of EGF resulted in EGFR dimerization (EC$_{50}$ = 14 nM [95% CI; 11, 19]),

407 while no increase in molecular brightness was observed in the presence of rHc/A, even with

408 upwards of 150 nM (EC$_{50}$ >150 nM) (**Figure S2; Table 1**), suggesting that rH$_C$/A has a high degree

409 of specificity for FGFRs.

410

DISCUSSION

412 In the current study, wt and mutant variants of the binding domain of BoNT/A (rH$_C$/A)

413 were evaluated in parallel with native FGF ligands possessing known FGFR subtype selectivity to

414 assess H$_C$/A's ability to induce FGFR receptor dimerization. This assessment was done using the

415 FCS & TIRF receptor dimerization assay, a method for measuring molecular associations between

416   proteins by quantifying the molecular brightness of a fluorophore determined through

417   fluctuations in the intensity (due to protein diffusion) within each pixel [49, 53]. This method

418   allows for receptor dynamics to be examined in live cells in response to various ligands, providing

419   continual observation under alternating conditions. The focus on the current investigation was

420   to study FGF receptor dimerization. However, for future studies, this method can potentially

421   provide detailed information on ligand-receptor stoichiometry, cluster formation, and kinetics of

422   receptor endocytosis through the use of fluorescently labeled ligands. The advantage of the FCS

423   & TIRF receptor dimerization assay is that it allows real-time analysis of receptor interactions in

424   live cells as well as direct assessment of the spatial organization of receptor self-association

425   state(s) on the plasma membrane. This presents a new method for addressing biological

426   questions not accessible using conventional terminal endpoint approaches.

427   Utilizing the FCS & TIRF receptor dimerization assay, dimerization of FGFRs and EGFR were

428   monitored in live neuronal cells in the presence of native and novel receptor ligands, such as the

429   binding domain of BoNT/A, rH_C/A. Consistent with other reports [64], low level of HaloTag FGF

430   receptor dimerization was observed in the absence of receptor ligand. The analysis was done

431   with cells expressing approximately 100,000-150,000 tagged FGF receptors per cell to minimize

432   potential effects associated with different densities of receptor on the plasma membrane. The

433   observed increase in molecular brightness with increased concentration of ligand was due to

434   dimerization rather than polymerization. This is because the assay exclusively measures the

435   population of receptors with normalized brightness values indicative of monomer or dimer states

436   (**Figure 2**). The fact that a plateau of normalized brightness at ~2 (or dimer species) was observed

437   for the non-clustering molecular brightness may indicate that these cells have been saturated

438   with ligand to the point that the endocytosis machinery cannot recycle the receptors fast enough

439   to clear dimeric complexes within the assay timeframe.

440   The EC_{50} values for FGF ligands dimerizing their cognate high affinity receptors ranged

441   from 17-19 nM (95% CI; 8, 35), which is consistent with what has been reported previously for

442   FGFR ligand binding and signaling [24, 35, 39, 65-68], while the EC_{50} values for rH_C/A were in the

443   rank order FGFR3c (27 nM [95% CI; 18, 41]) > FGFR2b (70 nM [95% CI; 59, 82]) > FGFR1c (163 nM

444   [95% CI; 123, 203]), suggesting that BoNT/A has preferential binding to the FGFR3c receptor

445   subtype. The cellular expression for each receptor subtype was similar (10,000-15,000 counts per

446   cell) and the EC_{50} value for the native ligands was similar for different FGFR subtypes, while the

447   EC_{50} value for H_C/A differed. Keeping in mind that this is an artificial system, these results support

448   a model where FGFR is part of the specific binding and, potentially, internalization of BoNT/A in

449   neuronal cells. Whether binding of BoNT/A to FGFRs results in pharmacological effects in vivo

450   and in patients is unclear, and it should be kept in mind that clinical toxin doses are in the pM

451   range. Interestingly, unlike most FGFs which are specific to either the b or c isoform of the

452   different receptor subtypes, rH_C/A dimerized both b and c isoforms, suggesting that H_C/A has the

453   potential to effect a large number of cell types that express FGFRs [38, 39, 41, 69, 70]. rH_C/A did

454   not dimerize EGFR at the highest concentration tested here (150 nM) (EC_{50} >150 nM), which

455   indicates rH_C/A's specificity for FGFR over other tyrosine kinase growth factor receptors

456   (summary of the results shown in **Table 1**). Whether FGFRs can facilitate uptake of BoNT/A in

457   other cell types and whether other BoNT serotypes can also bind to FGFRs remains to be further

458   explored.

459   The observation that gangliosides increase rH_C/A-mediated dimerization of FGFR3c, while

460   an rH_C/A variant wherein the residues important for GT1b binding have been mutated

461   (W1266L;Y1267S [16]) showed reduced ability to dimerize, suggests that GT1b and possibly other

462   gangliosides could act as co-receptors for FGFR to facilitate binding to BoNT/A. Although it is

463   unclear whether binding to FGFRs directly triggers internalization of BoNT/A, it is quite likely since

464   binding of FGF native ligands is known to trigger FGFR internalization via mechanisms involving

465   interactions with extended synaptotagmin-like protein, E-Syt2 [71, 72]. This presents a potential

466   evolutionary link between binding of BoNTs to FGFRs and synaptotagmin, the receptor for

467   BoNT/B [73, 74], BoNT/D [75, 76], and BoNT/G [77, 78]. Perhaps BoNT/A binds indirectly to

468   synaptotagmin via its interaction with FGFRs and that the binding of BoNT/A to FGFRs has evolved

469   from an interaction of BoNTs with synaptotagmin.

470   The specific binding site on H_C/A for SV2 has been identified as an exposed beta-strand

471   loop in the center of the binding domain [19, 20, 22, 79]. The observation that a variant of H_C/A

472   (T1145A;T1146A [20]) with mutations in residues important for SV2 binding shows reduced

473   ability to dimerize FGFR3c suggests that these residues, directly or indirectly, affect binding to

474   FGFRs. Perhaps the binding site for FGFR and SV2 overlap and binding of BoNT/A and SV2 is

475   sequential. However, it is also possible that the reduced binding affinity observed by the H_C/A

476   T1145A;T1146A variant may arise due to perturbations adjacent to the FGFR3c binding region of

477   H_C/A.

478   In summary, the data presented here shows that the binding domain of BoNT/A (rH_C/A)

479   binds to and dimerizes FGFRs in transfected neuronal PC-12 cells. These results further support

480   a model where FGFRs, in particular FGFR3c, which is the primary FGFR subtype expressed in the

23

481   nervous system [32, 58, 59], function as receptors for BoNT/A and perhaps other BoNT subtypes

482   and serotypes on neuronal cells. The potential pharmacological consequence of BoNT binding to

483   FGFRs, both with respect to BoNT neuronal selectivity and potency and potential receptor

484   mediated effects on neuronal and non-neuronal cell types, is being explored.

485

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488

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737

738   **S1 Figure.** Schematic representation of TIRF N&B analysis of halo tagged FGFR in PC-12 cells.

739   (**A, D**) Intensity images of ~ 1000 images per cell were collected on cells under (**A**) serum

740   starved conditions followed by treatment with (**D**) growth factor. (**B, E**) The intensity

741   fluctuations within each pixel of the collected images are used to calculate the average intensity

742   and brightness in a 2-D histogram. Regions of interest are selected to determine the population of

743   the monomers (red) and dimers (green). These different populations are represented as an

744   overlay in the images (**C, F**).

745

746   **S2 Figure. rH_C/A does not induce EGFR dimerization.** BoNT/A binding domain (rH_C/A) (green)

747   does not dimerize EGFR (EC_{50} >150 nM), while the native ligand for EGFR, EGF (black) can

748   dimerize with high potency (EC_{50} = 14 nM (95% CI; 11, 19]). Points represent the average

749   normalized brightness values ±SD from greater than 30 cells collected on 4 different days.
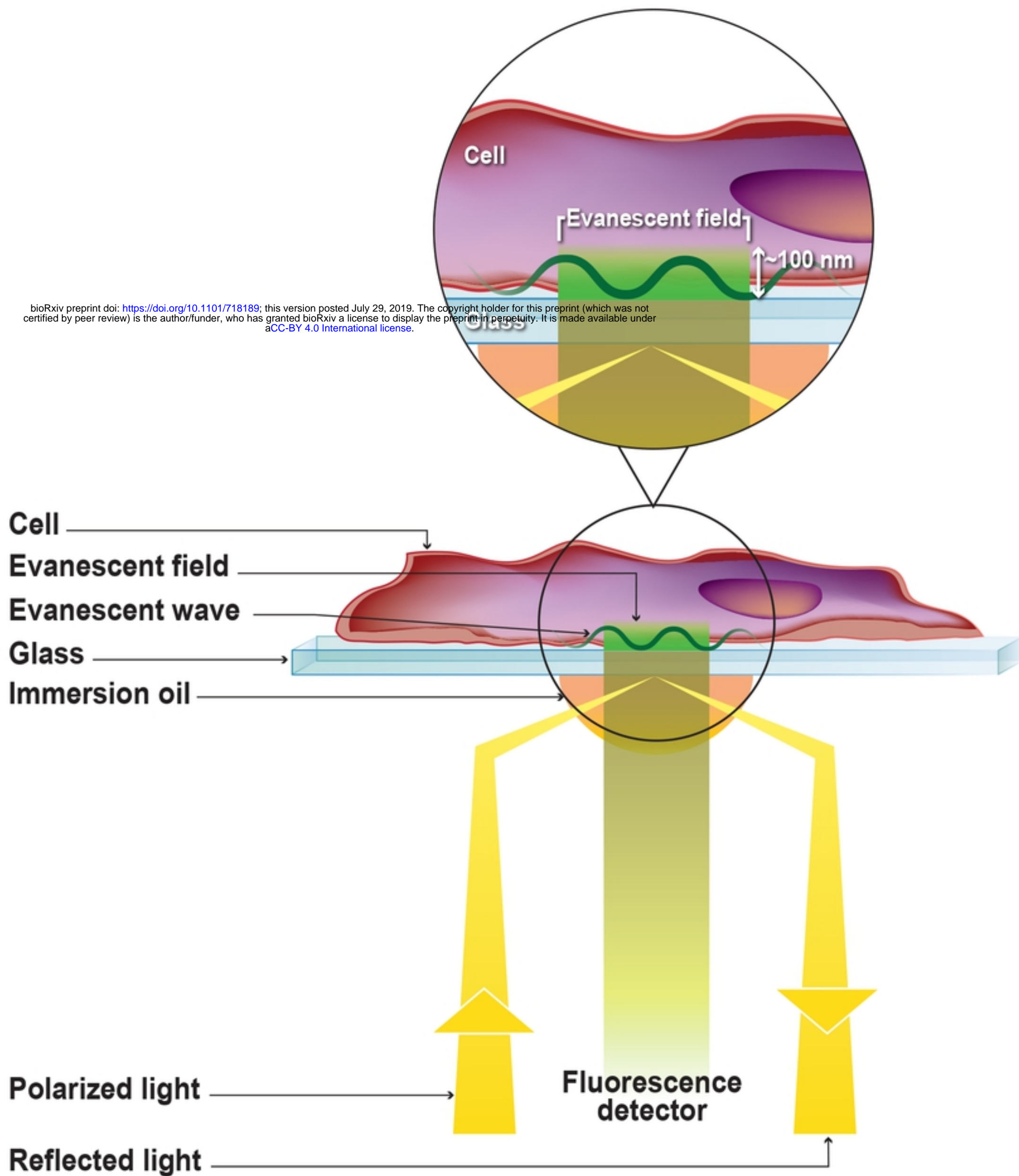
Cell

Evanescent field

Evanescent wave

Glass

Immersion oil

Polarized light

Reflected light

Fluorescence detector

Figure 1

A

FGFR3 only   FGFR3 + 25 nM FGF9
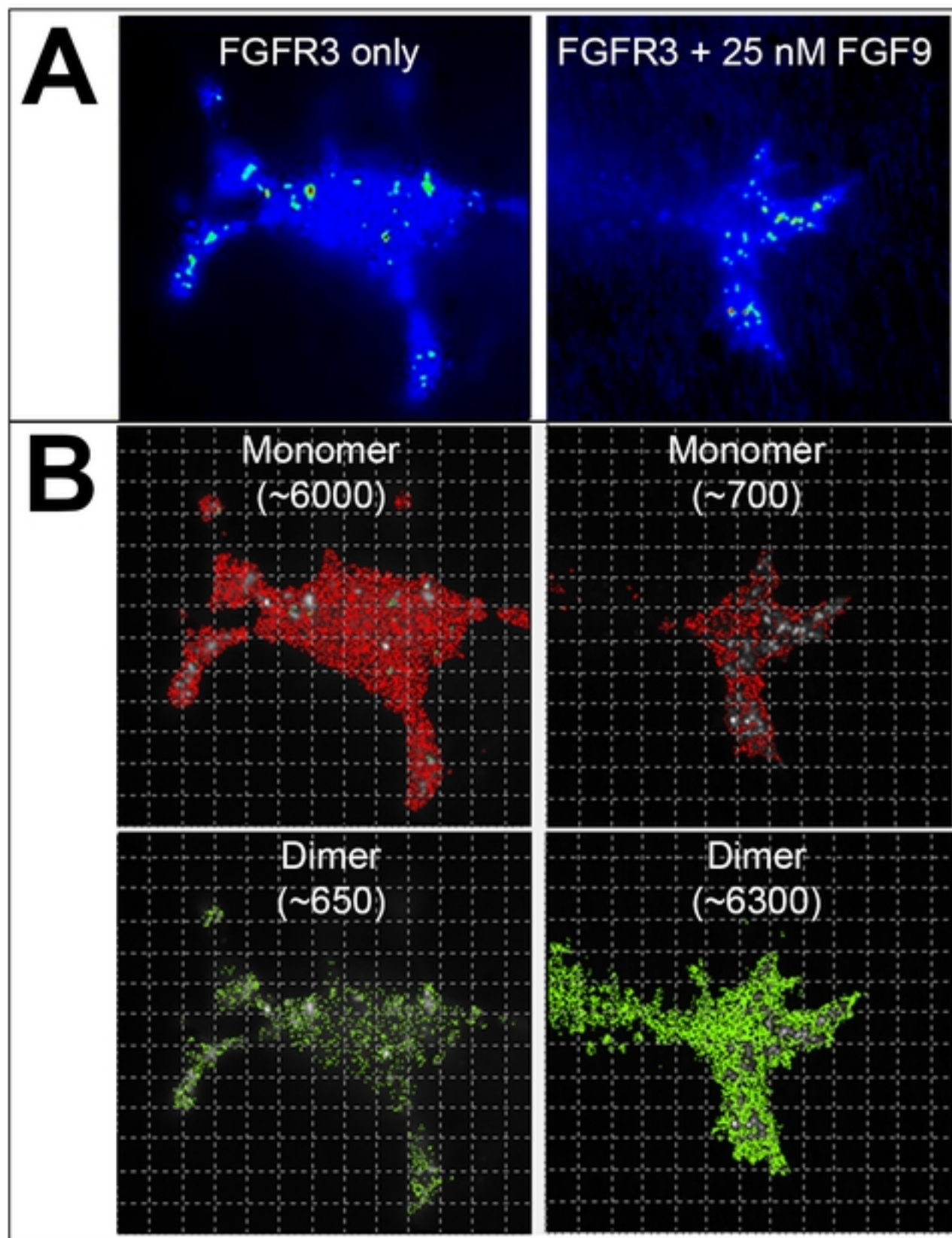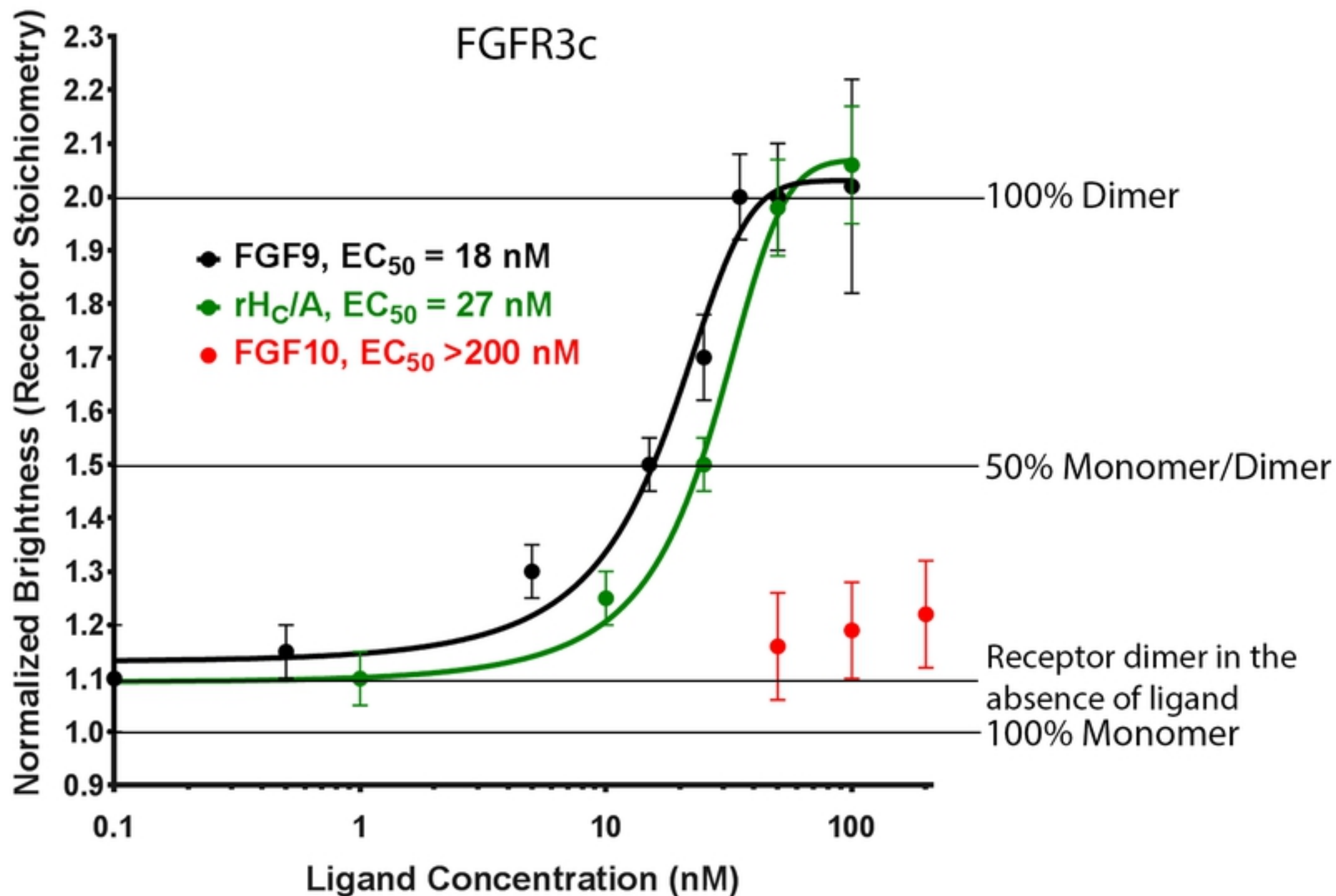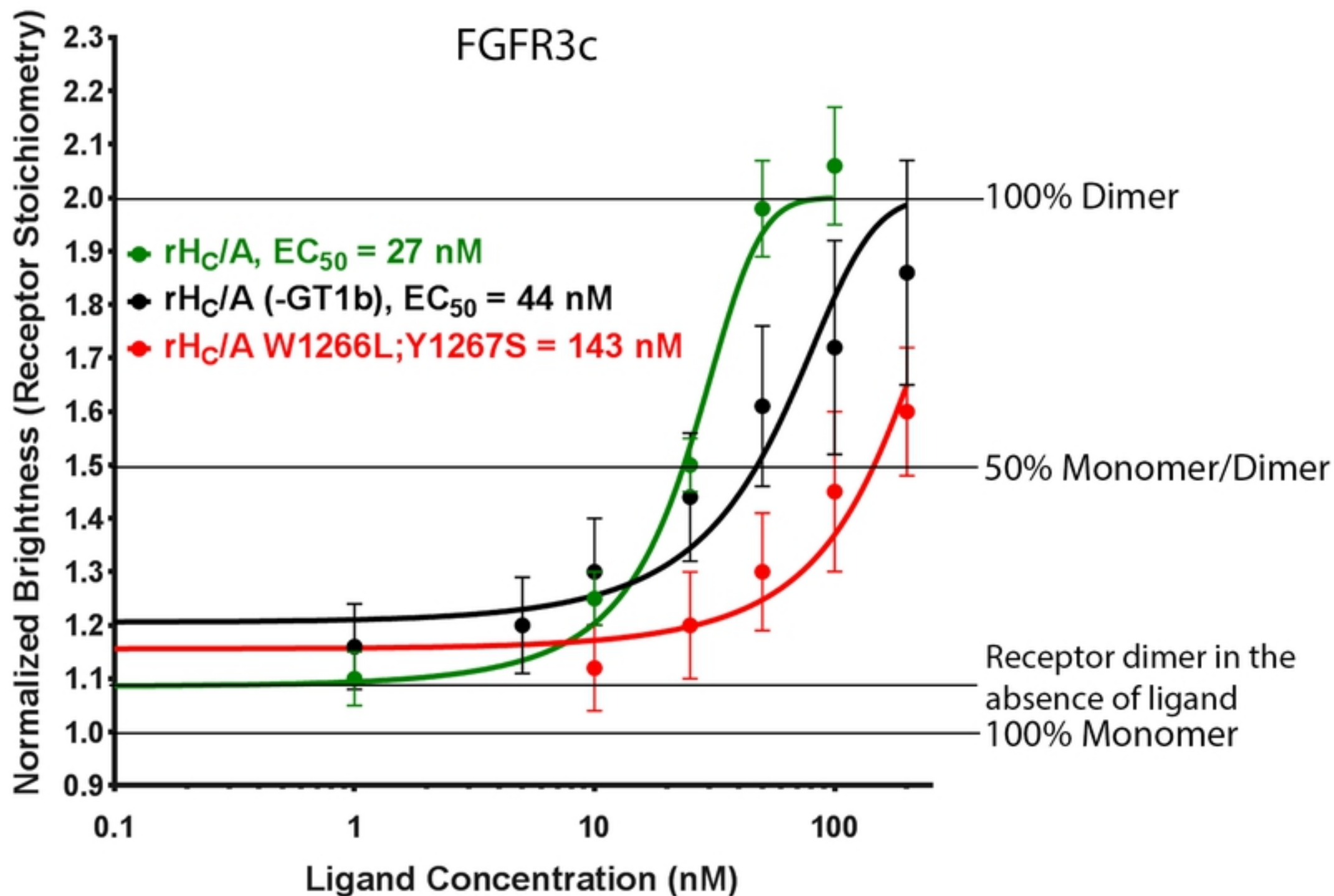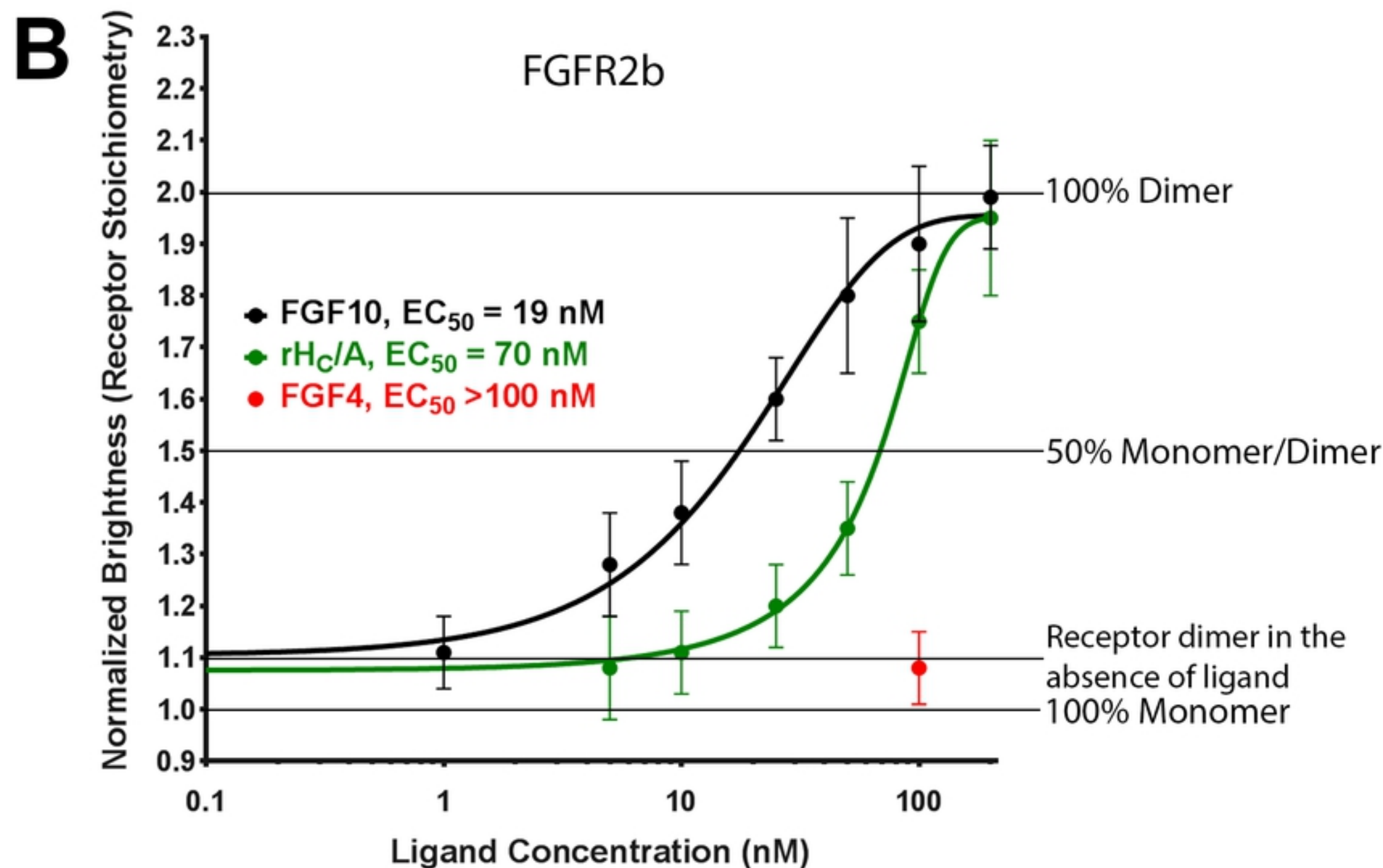
B

Monomer (~6000)   Monomer (~700)

Dimer (~650)   Dimer (~6300)

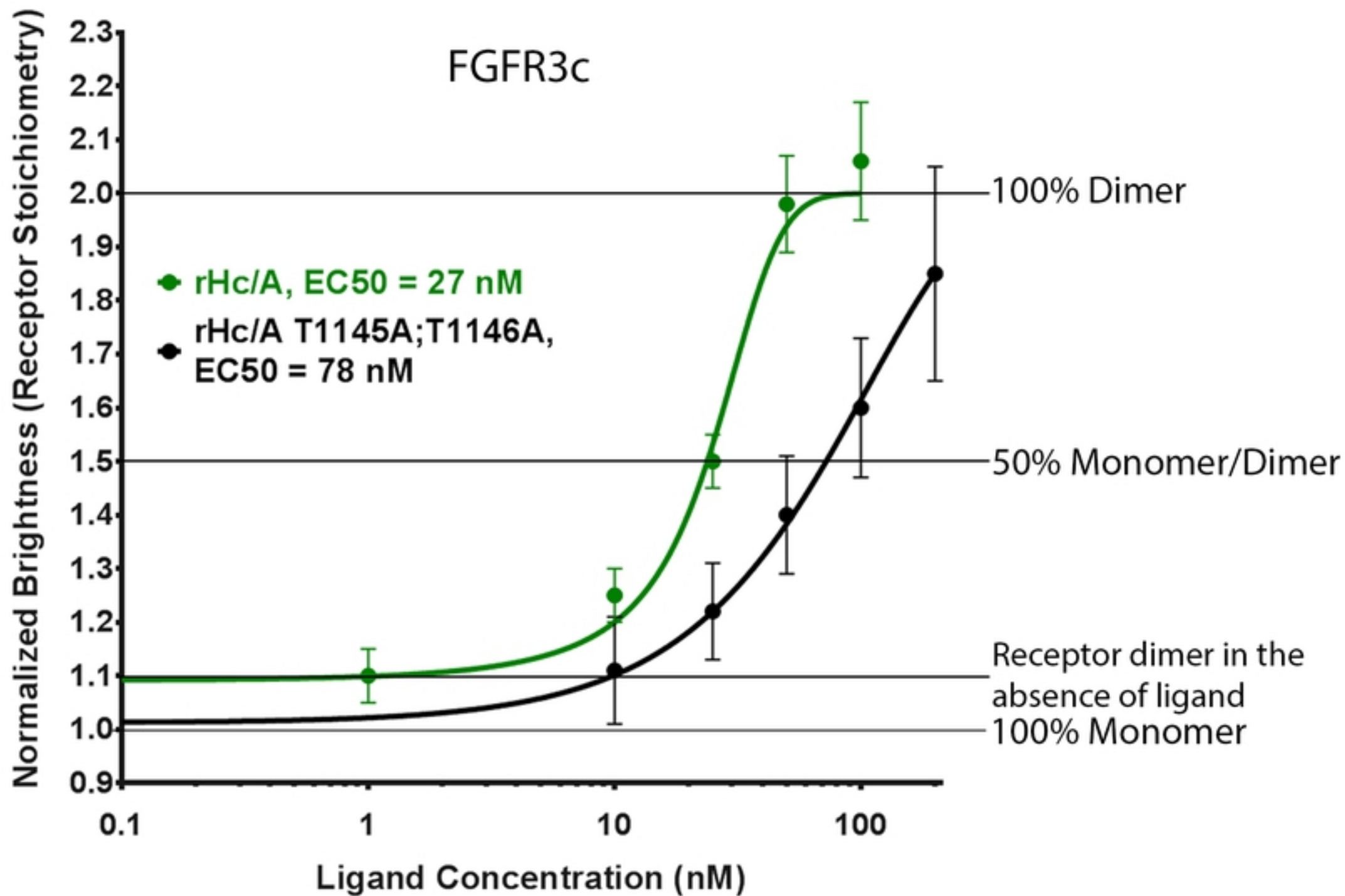Figure 2

Figure 3

Figure 4

A FGFR1c

- FGF4, EC_{50} = 17 nM
- rH_C/A, EC_{50} = 163 nM
- FGF10, EC_{50} >100 nM

100% Dimer
50% Monomer/Dimer
Receptor dimer in the absence of ligand
100% Monomer

Normalized Brightness (Receptor Stoichiometry)

Ligand Concentration (nM)

B FGFR2b

- FGF10, EC_{50} = 19 nM
- rH_C/A, EC_{50} = 70 nM
- FGF4, EC_{50} >100 nM

100% Dimer
50% Monomer/Dimer
Receptor dimer in the absence of ligand
100% Monomer

Normalized Brightness (Receptor Stoichiometry)

Ligand Concentration (nM)

Figure 5

Figure 6

Figure S1

Figure S2