Direct imaging of APP proteolysis in living cells

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It is now widely accepted that oligomers consisting of Aβ peptide are the cytotoxic species contributing mostly to the development of Alzheimer's disease. Aβ peptide production results from the specific proteolytic processing of the amyloid precursor protein (APP). Deciphering the factors governing the activity of the secretases responsible for the cleavage of APP is still a critical issue. Kits available commercially measure the enzymatic activity of the secretases from cells lysates, *in vitro*. By contrast, we have developed a prototypal rapid bioassay that provides visible information on the proteolytic processing of APP directly in living cells. APP was fused to a monomeric variant of the green fluorescent protein and a monomeric variant of the red fluorescent protein at the C-terminal and N-terminal, respectively. Changes in the proteolytic processing rate in transfected human neuroblastoma and rat neuronal cells were imaged with confocal microscopy as changes in the red/green fluorescence intensity ratio. The significant decrease in the mean red/green ratio observed in cells over-expressing the β-secretase BACE1 fused to a monomeric blue fluorescent protein confirms that the proteolytic site is still accessible. Specific siRNA was used to evaluate the contribution of endogenous BACE1. Interestingly, we found that the degree of proteolytic processing of APP is not completely homogeneous within the same single cell, and that there is a high degree of variability between cells of the same type. We were also able to follow with a fluorescence spectrometer the changes in the red emission intensity of the extracellular medium when BACE1 was overexpressed. This represents a complementary approach to fluorescence microscopy for rapidly detecting changes in the proteolytic processing of APP in real time. Moreover, we obtained a quantitatively robust estimate of the changes in the red/green ratio for the above conditions by using a flow cytometer able to simultaneously excite and measure the red and green fluorescence. Our novel approach lay the foundation for a bioassay suitable to study the effect of drugs or particular conditions, to investigate in an unbiased way the the

proteolytic processing of APP in single living cells in order, and to elucidate the causes of the variability and the factors driving the processing of APP.

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Abstract

Alzheimer's disease is a multifactorial disorder caused by the interaction of genetic, epigenetic and environmental factors. The formation of cytotoxic oligomers consisting of Aβ peptide is widely accepted as being one of the main key events triggering the development of Alzheimer's disease. Aβ peptide production results from the specific proteolytic processing of the amyloid precursor protein (APP). Deciphering the factors governing the activity of the secretases responsible for the cleavage of APP is still a critical issue. Kits available commercially measure the enzymatic activity of the secretases from cells lysates, *in vitro*. By contrast, we have developed a prototypal rapid bioassay that provides visible information on the proteolytic processing of APP directly in living cells. APP was fused to a monomeric variant of the green fluorescent protein and a monomeric variant of the red fluorescent protein at the C-terminal and N-terminal (mChAPPmGFP), respectively. Changes in the proteolytic processing rate in transfected human neuroblastoma and rat neuronal cells were imaged with confocal microscopy as changes in the red/green fluorescence intensity ratio. The significant decrease in the mean red/green ratio observed in cells over-expressing the β-secretase BACE1, or the α-secretase ADAM10, fused to a monomeric blue fluorescent protein confirms that the proteolytic site is still accessible. Interestingly, we found that the degree of proteolytic processing of APP is not completely homogeneous within the same single cell, and that there is a high degree of variability between cells of the same type. We were also able to follow with a fluorescence spectrometer the changes in the red emission intensity of the extracellular medium when BACE1 was overexpressed. This represents a complementary approach to fluorescence microscopy for rapidly detecting changes in the proteolytic processing of APP in real time. In order to allow the discrimination between the α- and the β-secretase activity, we have created a variant of mChAPPmGFP with a mutation that inhibits the α-secretase cleavage without perturbing the β-secretase processing. Moreover, we obtained a quantitatively robust estimate

51   of the changes in the red/green ratio for all the above conditions by using a flow cytometer able

52   to simultaneously excite and measure the red and green fluorescence. Our novel approach lays

53   the foundation for a bioassay suitable to study the effect of drugs or particular conditions, to

54   investigate in an unbiased way the proteolytic processing of APP in single living cells, and to

55   elucidate the causes of the variability and the factors driving the processing of APP.

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Introduction

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71   In the last decades the incidence of Alzheimer's disease (AD) has risen due to an increase in

72   the lifespan, representing more and more a serious social problem. The first steps in the

73   pathological cascade involve protein quality control impairment and the failure of misfolded

74   proteins clearance mechanisms, with subsequent accumulation and oligomeritazion of

75   neurotoxic amyloid-β peptide. Aβ oligomers decrease the synaptic efficacy, activate microglia

76   and astrocytes causing an inflammatory response, alter the degree of tau's phosphorylation

77   leading to tangles, deposit in the brain blood vessels and cause neuronal loss and

78   neurotransmitter deficits(Selkoe and Hardy, 2016). The generation of amyloid-β peptide results

79   from the sequential cleavage of the amyloid precursor protein (APP) by β-secretase and γ-

80   secretase(Haass, 2004) (De Strooper, 2010). This process is in competition with an alternative

81   non-pathogenic pathway lead by the α-secretase(De Strooper, 2010). APP is a plasma

82   membrane glycoprotein whose original function remains unclear (Senechal, Larmet and Dev,

83   2006). The β-secretase and the other secretases have become obvious therapeutic targets for

84   drug research in AD (MacLeod, et al., 2015). Several strategies have mainly focused on the

85   inhibition of β-secretase and γ-secretase or, alternatively, on Aβ clearance. Unfortunately, none

86   of these pharmacological therapies has yet passed the phase II/III trial due to a lack of

87   encouraging results, or to the onset of adverse side effects (MacLeod, et al., 2015). Despite the

88   recent failures, significant hope still lies around the secretase targeting approach. With this

89   regard, the full understanding of the factors governing the rate of proteolytic processing of APP

90   is imperative. Commercial kits (Sigma-Aldrich, Abcam) to evaluate the activity of the β-

91   secretase BACE1 from human and animal samples are available. These assays use a specific

92   BACE1 substrate conjugated to two reporter molecules. In the uncleaved form, the fluorescence

93   of the reporter is quenched by the physical proximity of the other molecule. After the cleavage

94   by β-secretase, the two molecules are separated and a fluorescent signal can be acquired with

95   intensity proportional to the enzymatic activity. This type of kit is very efficient in detecting the

96   activity of BACE1 from purified samples, but remains an *in vitro* evaluation. By contrast, we

97   have developed a consistent bioassay to quantify in an unbiased way the the proteolytic

98   processing of APP in single living cells. The key element of our assay is a chimeric construct of

99   APP with two different fluorescent proteins fused at the opposite terminals. Changes in the

100 proteolytic processing of APP are seen as changes in the intensity ratio of the two fluorescent

101 protein tags. We demonstrate that our mChAPPmGFP construct is efficiently recognized and

102 cleaved by both α- and β-secretase. The activity of each individual secretase can be

103 discriminated by using specific siRNAs or, alternatively, by introducing specific mutations at the

104 cleavage sites of APP. Here, we have created a mutated variant of mChAPPmGFP that can be

105 cleaved almost exclusively by β-secretase (mChAPP^{P1}mGFP). The mutation, a Lys to Val

106 substitution at position 612 of APP695, just before the α-secretase cleavage site (this position is

107 called P1), has already been used to verify the sequence specificity of α-secretase

108 cleavage(Sisodia, 1992) . Sisodia tested a series of mutants at the P1 position, and found by

109 immunoprecipitation studies that only one, the Lys to Val substitution, prevents the α-secretase

110 cleavage.

111 Finally, we obtained a quantitatively robust validation of our bioassay by using a flow cytometer

112 able to simultaneously excite and measure the emission intensity of the two fluorescent

113 proteins.

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Materials and Methods

Cloning

To generate the fusion constructs of mChAPPmGFP and mChAPP770mGFP we used

APP695 and APP770 contained in the vector pCMV-AC-GFP (Origene, Rockville MD, USA).

First, we inserted new restriction sites (NheI, AscI) after the signal peptide using the GeneArt

Site-Directed Mutagenesis Kit (ThermoFisher Scientific), using

GCCGCCTGGACGGCTCGGGCGGCTAGCCAGGGCGCGCCTCTGGAGGTACCCACTGATG

G as forward primer, and

CCATCAGTGGGTACCTCCAGAGGCGCGCCCTGGCTAGCCGCCCGAGCCGTCCAGGCGGC

as reverse primer. The mCherry sequence was amplified from its original plasmid (pmCherry-C1

vector, Clontech) with a PCR reaction (forward primer:

TAAGCAGCTAGCATGGTGAGCAAGGGCGAGGAG, reverse primer

TGGCGCGCCTGTTCCACGACTCTTGTACAGCTCGTCCATG) adding the restriction

sequences for the enzymes NheI and AscI. After digestion, the sequence of mCherry was

inserted at the N-term of APP. In order to substitute the dimerizing TurboGFP present in the

pCMV-AC-GFP vector with the monomeric green fluorescent protein mTagGFP2, mCherry-

APP695 and mCherry-APP770 were excised from their original plasmid using the restriction

enzymes SgfI and NotI and inserted in the plasmid pCMV6-AN-mGFP (commercial name of

mTagGFP2, Origene) to obtain the final fusion constructs mCherry-APP695-mGFP and

mCherry-APP770-mGFP. The QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent)

was used to mutagenize the mChAPPmGFP construct in order to create a new plasmid

(mChAPP[P1]mGFP) carrying the P1 mutation, a Lys to Val substitution at P1 position (amino

acid 612 of APP695), that is the amino acid located before the α-secretase cleavage

site(Sisodia, 1992) (forward primer: GAAGTTCATCATCAAGTATTGGTGTTCTTTGC, reverse

140   primer: GCAAAGAACACCAATACTTGATGATGAACTTC).

141   To generate the fusion construct Bace1-mBFP and HA-Bace1-mBFP, we started from Bace1

142   contained in the vector pCMV6-ENTRY (NM_012104, Origene, Rockville, MD, USA). We

143   opened the vector using the restriction enzymes NotI and SacII. mBFP (original name

144   mTagBFP2) was extracted with a PCR reaction from the plasmid pmTagBFP2-N1(Subach, et

145   al., 2011) (forward primer: TAAGCAGCGGCCGCGAATGAGCGAGCTGATTAAGG, reverse

146   primer: CCGAGTCCGCGGTTAATTAAGCTTGTGCCCCAG) to add the restriction sequences of

147   the enzymes NotI and SacII. After digestion, the sequence of mBFP was inserted in the plasmid

148   of Bace1 previously opened with the same enzymes, to obtain the final fusion construct Bace1-

149   mBFP. With a site-directed mutagenesis reaction (ThermoFisher Scientific) we added the

150   restriction sites for NheI and AscI at the N-term of Bace1-mBFP (forward primer:

151   GGAGTGCTGCCTGCCCACGGCGCTAGCCAGGGCGCGCCtACCCAGCACGGCATCCGGCT

152   G, reverse primer

153   CAGCCGGATGCCGTGCTGGGTAGGCGCGCCCTGGCTAGCGCCGTGGGCAGGCAGCACT

154   CC). We opened the plasmid of Bace1-mBFP and inserted the annealed oligos containing the

155   sequence for the HA tag (forward oligo: CTAGCTATCCGTACGACGTACCAGACTACGCAGG,

156   reverse oligo: CGCGCCTGCGTAGTCTGGTACGTCGTACGGATAG). After ligation we obtained

157   the plasmid of the construct HA-Bace1-mBFP.

158   ADAM10mBFP was created by the fusion of the mTagBFP2 gene downstream of the ADAM10

159   gene contained in the pRK5M-ADAM10 construct (Addgene). The final part of the sequence

160   coding for ADAM10 was mutagenized in order to introduce two new restriction sites, unique

161   within the entire sequence of the plasmid. The site-directed mutagenesis was performed with

162   the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), using

163   GAGGACCTGCTGCGTACGGTCGCGCGCGCTTGGCCGCCA as forward primer and

164   TGGCGGCCAAGCGCGCGCGACCGTACGCAGCAGGTCCTC as reverse primer, containing

165   the restriction sequences for the BsiWI e BssHII enzymes. The mTagBFP2 sequence was

166   amplified from its original plasmid (pmTagBFP2-N1, Addgene) with a PCR reaction (forward

167   primer GAGCGTACGATGAGCGAGCTGATTAAGGAG, reverse primer

168   GTAGCGCGCTTAATTAAGCTTGTGCCCCAG, the forward primer containing the BsiWI

169   restriction site and the reverse the BssHII one). After digestion, the sequence of BFP2 was

170   inserted at the C-terminal of ADAM10.

171   apAPPha was created from mChAPPmGFP using the restriction sites for NheI and AscI at the

172   N-term. First, we opened the plasmid with these enzymes and inserted the annealed oligos

173   containing the sequence for the genetic tag AP (forward oligo:

174   CTAGCGAGGGCCTGAACGATATCTTCGAGGCCCAGAAGATCGAGTGGCACGAGAGTGGre

175   verse oligo:

176   CGCGCCACTCTCGTGCCACTCGATCTTCTGGGCCTCGAAGATATCGTTCAGGCCCTCG).

177   Afterwards, we opened the plasmid with the restriction enzimes NotI and SacII in order to

178   substitute mGFP with the annealed oligos containing the sequence for the genetic tag HA

179   (forward oligo:

180   GGCCGCTCGAGTATCCGTACGACGTACCAGACTACGCAGTTTAAACCCGC,

181   reverse oligo: GGGTTTAAACTGCGTAGTCTGGTACGTCGTACGGATACTCGAGC).

Cell cultures and transfection

183   Human SH-SY5Y neuroblastoma cells (A.T.C.C. Manassas, VA, USA) were cultured in

184   Dulbecco's Modified Eagle's Medium (DMEM) F-12 supplemented with 10% FBS, 1%

185   penicillin/streptomycin solution. HEK cells were cultured in DMEM Glutamax supplemented with

186   10% FBS, 1% penicillin/streptomycin solution. Primary rat hippocampal neurons were obtained

187   after dissociation of hippocampi purchased from Brainbits, UK. Neurons were plated on

188   coverslips coated with poly-L-ornithine and grown in Neurobasal medium supplemented with 2%

189   B27, 0,5 mM glutamine, 12,5 mM glutamate and 0,1 % penicillin/streptomycin. All the cell types

190   were cultured in a humidified chamber at 5% CO_2 and 37°C. Neuroblastoma SH-SY5Y cells

191   were transfected with Fugene HD (Promega, USA) 24-48 h before imaging. HEK cells were

192   transfected with Lipofectamine 2000 (ThermoFisher Scientific) 24-48 h before imaging. To

193   transfect hippocampal neurons DNA was mixed with Lipofectamine 2000 in 0,5 ml of

194   Neurobasal supplemented with 0,5 mM glutamine at 37°C in 5% CO_2 for 60 min. Afterwards

195   neurons were rinsed with Neurobasal, transferred to the original medium at 37 °C 5% CO_2 and

196   imaged after 24-48 h. apAPPha was co-transected with BirA, a biotin ligase that specifically

197   biotinylates the AP tag . Biotin was added to the cell medium (final concentration 10 μM) 8 hours

198   after transfection and 12 hours before imaging(Howarth, et al., 2005). siRNA Silencer®

199   Validated siRNA against BACE1 (105154) and *Silencer*® Negative Control siRNA were

200   purchased from ThermoFisher. Supplementary Figure S5 shows the silencing efficiency of

201   Bace1-siRNA.

202   **Cell imaging**

203   Live cell imaging was performed on a Nikon Eclipse TE300 C2 LSCM (Nikon, Japan) equipped

204   with a Nikon 60x immersion oil objective (Apo Plan, NA 1.4). Cells were growth on 18 mm

205   coverslips and mounted on a custom chamber containing 1 ml of Leibovitz's medium

206   (ThermoFisher). For immunolabelling, cells were fixed with 4% PFA, rinsed with PBS (+$MgCl_2$

207   0,5 mM, +$CaCl_2$ 0,8 mM) and permeabilized with 0,075% Triton X. After rinsing with PBS and

208   blocking with 4% BSA PBS, cells were labelled with primary (1:1000 rabbit anti-HA ab9110 -

209   Abcam, UK-) and secondary antibodies (goat anti-rabbit Alexa 488,–ThermoFisher, diluted at

210   1:500), or streptavidin Alexa 568 (ThermoFisher diluted at 1:500), diluted in PBS with 4% BSA.

211   After rinsing again with PBS, coverslips were mounted on a glass slide and imaged with LSCM,

212   equipped with Coherent CUBE (diode 405 nm), Melles Griot (Argon 488 nm) and Coherent

213   Sapphire (Sapphire 561 nm) lasers. Emission filters for imaging were 452/45 nm, 514/30 nm

214   and 595/60 nm. For surface labeling, living SH-SY5Y cells were growth on 18 mm coverslips,

215   rinsed with PBS and incubated with primary antibodies (1:100 rabbit anti-mCherry ab167453, or

216 1:1000 rabbit anti-HA ab9110 -Abcam, UK-) diluted in PBS with 4% BSA for 30 minutes on ice,

217 to inhibit endocytic events. After rinsing with PBS,cells were incubated with secondary

218 antibodies (1:500 goat anti-rabbit Alexa 405) diluted in PBS with 4%BSA for 15 minutes. After

219 rinsing again the coverslips, cells were mounted on a custom chamber filled with Leibovitz's

220 medium and imaged with the same LSCM configuration mentioned above.

221 Western blotting

222 Human SH-SY5Y neuroblastoma cells were cultured in 6 wells plates. 24 h after transfection,

223 the extracellular medium was recovered and the cells were lysed using 300 µl of PBS and

224 Laemmli buffer. Lysed cells were collected in safe lock 1,5 ml tubes and immersed in boiling

225 water for 5 minutes, then sonicated two times for 15 seconds and boiled again for 5 minutes.

226 The recovered medium was centrifuged at 10000 rpm for 5 min in order to remove cell debrees

227 and other impurity, and then treated as the cell lysate. Samples were loaded in pre-casted

228 polyacrylamide gels (ThermoFisher Scientific, MA USA) and blotted on PVDF membranes

229 (ThermoFisher Sicnetific, MA USA). Membranes were blocked in 5% milk in PBST overnight at

230 4°C or 1 h at room temperature. After blocking, membranes were rinsed three times with PBST

231 and incubated at room temperature with primary antibody (in PBST with 4% BSA) for 1 h and for

232 an additional hour with secondary anti-rabbit antibody conjugated to horseradish peroxidase

233 (1:20000). The primary antibodies were: rabbit anti-mCherry (1:1000, Abcam, UK, ab167453),

234 rabbit anti-APP (1:200, Abcam ab15272), rabbit anti-vimentin (1:2500, Abcam ab92547

235 EPR3776) mouse anti-aβ (1:100, Abcam ab11132 DE2b4), mouse anti-GFP (1:1000 Abcam,

236 ab291 LGB-1). Membranes were rinsed with the HRP substrate Pico Dura (ThermoFisher

237 Scientific, MA USA) and imaged after 5 min with a Chemidoc MP system (Bio-Rad, CA USA). In

238 order to perform the stripping procedure, the blotted PVDF membranes were covered with

239 Seppro Stripping Buffer (Sigma Aldrich, MO USA) and incubated for 5-10 min at room

240 temperature. The stripping buffer was then discarded and the incubation was repeated with

241 fresh Seppro buffer for 5-10 min. After discarding the buffer, membranes were washed for two

242 times with PBS for 10 minutes, then washed for two times with TBST for 5 minutes and blocked.

Fluorescence spectroscopy

244 Fluorescence spectra from 585 nm to 700 nm were acquired at 25 °C in a 10 ×10 mm quartz

245 cuvette using a Perkin-Elmer LS 55 spectrofluorimeter (Waltham, MA, USA) equipped with a

246 thermostated cell holder attached to a Haake F8 water bath (Karlsruhe, Germany). Excitation

247 wavelength was 550 nm.

FACS

249 Cells were harvested after mild trypsin treatment and centrifuged for 5 min at 330 g; the

250 obtained pellets were washed and re-suspended in PBS. We cannot exclude that the treatment

251 with trypsin might affect the integrity of the ectodomain of mChAPPmGFP; however, all the

252 conditions of transfection tested would have been affected equally without influencing the

253 conclusion of our experiments. Flow cytometry was performed by using a S3 flow cytometer

254 (Bio-Rad) equipped with 488 and 561 nm diode-pumped solid-state lasers. mGFP was excited

255 using the 488 nm laser, and the emitted fluorescence was collected through a 586/25 nm band-

256 pass filter; mCherry was excited with the 561 nm laser and its fluorescence was collected

257 through a 615/25 nm band-pass filter. Data were analysed using *ProSort* software (Bio-Rad), by

258 dividing the scatter plot of mGFP versus mCherry into two equally populated regions under

259 control conditions. The cellular debris was excluded from the quantification by gating cells on

260 the Forward scatter area/Side scatter area (FSC/SSC) graph, while doublets were excluded by

261 gating the cells on the Forward scatter height/ Forward scatter area (FSC/FSC) graph; at least

262 10000 events were acquired for each sample.

263

Results

mChAPPmGFP is correctly targeted to the plasma

membrane

APP695, the isoform mainly expressed in the central nervous system (Nalivaeva and Turner, 2013), was fused to a monomeric variant of the green fluorescent protein (mGFP) at the C-terminal and to a monomeric red fluorescent protein (mCherry) at the N-terminal (Fig. 1). In this way the extremities of APP situated at the opposite sides of the plasma membrane are labeled with two different fluorescent proteins. Once the N-term mCherry containing domain is cleaved, the fluorescence intensity ratio between the red and green signals changes. It is thus possible to monitor in living cells a higher or lower processing of APP in function of the ratio shift. From herein, we will refer to this chimeric construct bearing the APP695 isoform as to mChAPPmGFP. The same procedure was repeated to obtain the fusion construct of APP770 (mChAPP770mGFP), an alternative isoform expressed more ubiquitously (Zheng and Koo, 2011).

mCherry was fused immediately after the signal peptide, which is necessary to target APP to the plasma membrane, and away from the site recognized and cleaved by the β-secretase . In order to test if mChAPPmGFP is correctly targeted to the plasma membrane, we performed a surface immunolabelling of transfected human SH-SY5Y neuroblastoma cells with a primary anti-mCherry antibody (Fig. 2a). Confocal laser scanning microscopy (CLSM) imaging reveals a good degree of labeling only in cells expressing mChAPPmGFP, confirming that mCherry is correctly folded and present on the surface of the cell. We observed red clusters not colocalising with anti-mCherry, thus indicating their intracellular localization.

286   To confirm that the full-length chimeric mChAPPmGFP protein was expressed, we examined

287   the colocalization of mCherry and mGFP in neuroblastoma cells (Fig. 2 b-d) and rat

288   hippocampal neurons (Fig. 2 e-g). The toolbox available with the JACoP plug-in(Bolte and

289   Cordelieres, 2006) under the ImageJ software(Schneider, Rasband and Eliceiri, 2012) was used

290   to quantify the degree of colocalization . We found that in most cells the plot of the pixel intensity

291   of the green against that of the red channel (scatterplot) follows a linear relationship, with an

292   average Pearson's coefficient (an estimate of the quality of the linear relationship between the

293   two signals with 1 standing for complete positive correlation) of 0.91. We also found an average

294   Manders' overlap coefficient(Manders, et al., 1992) M1 (indicating the fraction of mCherry

295   overlapping with mGFP) of 0.97 and an M2 (fraction of mGFP overlapping mCherry) of 0.85.

296   The measured Costes' randomization P-value(Costes, et al., 2004) of 100% excludes that the

297   co-localization of pixels is due to chance. An additional method such as Li's intensity correlation

298   analysis(Li, et al., 2004) further supports a very good degree of   colocalization, indicating an

299   abundant presence of full-length mChAPPmGFP. We recurrently observed small red fluorescent

300   punctae, at which did not correspond a comparable level of green fluorescence intensity. Similar

301   data were obtained for mChAPP770mGFP (Supplementary Fig. S1).

302   Due to their size and consequent steric hindrance, the two fluorescent tags could alter the

303   distribution features of APP. However, we did not observe any significant difference in the

304   overall cellular distribution after immunolabeling of an overexpressed construct of APP695

305   bearing two small tags (AP(Howarth, et al., 2005) and HA(Schembri, et al., 2007), apAPPha) at

306   the two opposite terminals (Fig. 2h-j). As previously reported(Collins, et al., 2010;Guo, et al.,

307   2012;Haass, et al., 2012), a large fraction of intracellular mChAPPmGFP is localized at the level

308   of the Golgi apparatus, in the perinuclear region (Supplementary Fig. S2). In agreement with an

309   elegant study investigating the subcellular localization of endogenous full length APP in

310   neurons(Guo, et al., 2012), mChAPPmGFP also shows partial co-localization with the

311   endoplasmic reticulum, but very little with early endosomes and lysosomes (Supplementary Fig.

312   S2). We find this study of particular relevance since it shows with a knock-out model that many

313   antibodies against APP commonly used for immunofluorescence experiments give rise to non-

314   specific labeling.

Fluorescence intensity ratio heterogeneity

316   We then measured the ratio between the red and the green fluorescence intensity in

317   neuroblastoma cells (Fig. 3 a) and rat hippocampal neurons (Fig. 3 b). The ratio image was

318   generated using the Ratio Plus available on ImageJ (Schneider, et al., 2012). Regions of

319   interest (ROIs) for the calculation of the mean ratio value per cell were chosen by using a

320   constant threshold value on the maximum projection of mGFP stacks. The ROIs were then

321   applied to the corresponding ratio image. The gain used for the acquisition of neuronal images

322   was different from that of neuroblastoma cells due to differences in the level of protein

323   expression. We observed a certain degree of intracellular heterogeneity in the red/green ratio,

324   with lower red/green ratio in the peripheral areas of the cells. In addition, we observed a

325   relatively high intercellular variability among the same population of transfected neuronal or

326   neuroblastoma cells.

327

mChAPPmGFP is cleavable by Bace1

329   In order to check if our fusion protein was correctly folded, recognized and cleaved, we co-

330   transfected SH-SY5Y (Fig. 4) and neuronal cells (Fig. 5) with mChAPPmGFP together with β-

331   secretase Bace1 fused with mTagBFP2 (mBFP) at the C-term. We found that overexpressed

332   BACE1 is aboundantly present on the plasma membrane (Supplementary Fig. S3). When

333   Bace1-mBFP cleaves mChAPPmGFP on the cell surface, the soluble fragment mCherry-sAPPβ

334   is released in the extracellular medium. We compared the mean red/green ratio in cells

335   expressing mChAPPmGFP with or without Bace1-mBFP (Fig. 4 a-c; Fig. 5). While we did not

336   find statistically significant differences in cells transfected with siRNA against Bace1 (although

337   an unpredicted minor reduction in the ratio is visible), the overexpression of Bace1-mBFP

338   dramatically decreased the ratio. Although APP might undergo a major processing by α-

339   secretase under physiological conditions, the contribution of the latter is negligible when APP is

340   overexpressed with BACE1. Cells transfected with mChAPP770mGFP showed a comparable

341   trend.

342   Equivalent results were obtained when performing western blots of both cell lysate and

343   extracellular medium of SH-SY5Y cells co-transfected with mChAPPmGFP and Bace1-mBFP or

344   Bace1-siRNA, using anti-mCherry as primary antibody for chemoluminescent detection (Fig.

345   4d). In the lysate of cells co-transfected with mChAPPmGFP and Bace1-siRNA, two bands were

346   detected, one more intense at 152 KDa, corresponding to full-length mChAPPmGFP (expected

347   molecular weight 134 KDa), and one at 125 KDa, possibly corresponding to mCherry-sAPPα

348   (expected molecular weight 100 KDa) generated by endogenous α-secretase. For cells co-

349   transfected with mChAPPmGFP and Bace1-mBFP, the same two bands were visible, but in this

350   case the lower band was more intense, due to an increased production of mCherry-sAPPβ,

351   which has a molecular weight (expected molecular weight 94 KDa) comparable to mCherry-

352   sAPPα. Blotting of the extracellular medium revealed only one band at 121 KDa, corresponding

353   to the released mCherry-sAPPfragment. A considerably higher intensity band was found in the

354   case of cells co-transfected with Bace1 (increased production of mCherry-sAPPβ) as compared

355   to those co-transfected with Bace1-siRNA (mostly mCherry-sAPPα). Analogous results were

356   obtained for the cell lysate and the extracellular medium of cells transfected with

357   mChAPP770mGFP, with the bands being at higher molecular weights: ~163 KDa for the

358   uncleaved mChAPP770mGFP (expected molecular weight 142 KDa) and ~138 KDa for the

359   mCherry-sAPP770β (expected molecular weight 102 KDa).

360   Membrane stripping and reprobing with anti-GFP, anti-APP (directed against the N-terminal 44-

361   62 amino acids) or anti-Aβ (directed against amino acids 1-17 of Aβ) generated analogous

362   results. At long time exposures of the blot, bands corresponding to the C-terminal fragments of

363   mChAPPmGFP (C83/89-mGFP and AICD-mGFP) are visible (Supplementary Fig. S4). These

364   data corroborate further that our construct is recognized and processed by BACE1.

365   Furthermore, we collected and analyzed the supernatant media from different transfection

366   conditions of SH-SY5Y cells with a fluorescence spectrophotometer. In agreement with the

367   above results, we observed higher fluorescence intensity at 610 nm (maximum emission peak

368   for mCherry) in cells co-expressing mChAPPmGFP together with Bace1-mBFP (Fig. 4 e). This

369   result further supports that β-secretase recognizes and cleaves our fusion APP construct, which

370   is then in part released in the extracellular medium as mCherry-sAPPβ. Similar results were

371   found in HEK cells (Supplementary Fig. S5). Our data show an unexpected slight increase in

372   fluorescence intensity at 610 nm in the cells co-transfected with Bace1-siRNA compared to the

373   control condition. Overall these results suggest that the site cleaved by β-secretase is still

374   accessible and that is possible to follow the processing of APP as function of the ratio shift.

375   We finally validated our results with FACS flow cytometry, a statistically robust technique

376   capable of analyzing the red and green fluorescence intensity for a large number of single cells.

377   SH-SY5Y cells were co-transfected with mChAPPmGFP and Bace1-mBFP, or control siRNA, or

378   Bace1-siRNA. Two different areas in the red versus green fluorescence scatter plot were

379   arbitrarily chosen so that both areas contained the same number of cells co-transfected with

380   mChAPPmGFP and control siRNA (Fig. 6). The overexpression of Bace1-mBFP changed

381   significantly the ratio between the number of cells in the red and green areas. We observed only

382   a slight, although not significant, increase in the ratio when cells were co-transfected with siRNA

383   directed against Bace1. Comparable results were found using HEK cells (Supplementary Fig.

384   S6).

385

The P1 mutation inhibits the processing of APP by ADAM10

387 SH-SY5Y neuroblastoma cells were co-transfected with mChAPPmGFP or mChAPP^{P1}mGFP

388 and ADAM10mBFP in order to test whether the P1 mutation really inhibits the α-secretase

389 activity. We compared the mean red/green ratio in cells overexpressing ADAM10mBFP and

390 mChAPPmGFP with or without the P1 mutation (Fig. 7a-b). The red/green ratio was found to be

391 significantly higher in cells overexpressing mChAPP^{P1}mGFP compared to mChAPPmGFP,

392 showing not only that the ADAM10mBFP construct is proteolytically active, but also that the

393 presence of the P1 mutation strongly prevents the cleavage by α-secretase. These results were

394 validated by FACS flow cytometry (Fig. 7c and Fig S7). SH-SY5Y cells were co-transfected with

395 mChAPPmGFP or mChAPP^{P1}mGFP and ADAM10mBFP or mBFP. As above, two different

396 areas containing the same number of cells co-transfected with mChAPPmGFP and mBFP were

397 used to gate the scatter plots of cells co-transfected with the other conditions and calculate the

398 mean ratio between the number of cells in the red area over the number of cells in the green

399 area. The number of cells in the red area significantly increased with the overexpression of

400 mChAPP^{P1}mGFP compared to the wild-type, both in the presence of ADAM10mBFP or mBFP,

401 denoting an almost complete resistance to α-secretase proteolysis. Moreover, SH-SY5Y cells

402 were co-transfected with mChAPP^{P1}mGFP and BACE1mBFP in order to verify potential side-

403 effects of the P1 mutation on the β-secretase processing. The ratio between the number of cells

404 in the red and green areas resulting from the co-expression of mChAPP^{P1}mGFP and

405 BACE1mBFP is comparable with that from mChAPPmGFP and BACE1mBFP. This data

406 suggests that the variant of APP carrying the P1 mutation is hardly processed by ADAM10,

407 while still cleavable by Bace1.

408

Discussion

We have successfully demonstrated the proof of principle of a bioassay to evaluate the

processing of APP directly in single living cells, a less biased method compared to the *in vitro*

kits that are commercially available. To estimate the rate of processing of APP, we measured

the variation in the red/green fluorescence ratio emitted from cells overexpressing

mChAPPmGFP. We demonstrated that mChAPPmGFP is correctly folded and targeted to the

plasma membrane, and it is still cleavable by the β-secretase Bace1. We have noticed that a

similar approach has been tested in two previous works(Coughlan, et al., 2013;Villegas,

Muresan and Ladescu Muresan, 2014). In one of these papers, the residual C-terminal region of

APP770 (106 amino acids) carrying the Swedish mutation was fused with two different

fluorescent proteins (DsRed2 and EGFP) at the opposite terminal sides(Coughlan, et al., 2013).

By contrast, our mChAPPmGFP construct is generated from the full length APP695 and

contains the original signal-peptide sequence that is required for targeting the newly

synthesized protein to the plasma membrane. Furthermore, in order to avoid oligomerization

events, we used two monomeric fluorescent proteins, mCherry and mGFP. These two

fluorescent proteins have comparable maturation times of approximately 10-15 minutes(Shaner,

et al., 2004;Subach, et al., 2008), thus limiting the rise of artifacts due to different maturation

events. In agreement with the fact that the mCherry and the mGFP tags are kept more than 10

nm apart by the plasma membrane, we did not observe any Foster resonance energy transfer

(FRET), preserving therefore the linearity of the red/green ratio with respect of the amount of

cleaved APP (Supplementary Fig. S8). In the other interesting work(Villegas, et al., 2014),

APP695 was fused to CFP and YFP at the two opposite terminals, with CFP correctly positioned

after the signal peptide, to study the localization of APP fragments and the trafficking processes

occurring intracellularly. The authors found that the presence of the tags did not alter the

processing of APP by the secretases, and that some intracellular N-terminal fragments –

434   possibly corresponding to our observed red punctae - do not colocalize with full length APP or

435   C-terminal fragments, in complete agreement with our data. Importantly, the dual tagged APP,

436   at low level of expression, showed a subcellular distribution and a proteolytical profile highly

437   comparable to that of endogenous APP.

438   Interestingly, our results show that cells of the same type (both neuronal and neuroblastoma

439   cells) display different ratios of red/green fluorescence intensity, suggesting that the processing

440   rate of APP is highly variable even within the same population. Furthermore, a certain level of

441   heterogeneity in the red/green intensity ratio is also found within the same cell, indicating that

442   the activity of the proteases can vary in different districts of the cell. This finding is in agreement

443   with previous results showing that there are differences in the processing of APP depending on

444   its targeting on the plasma membrane of highly polarized cells (Haass, et al., 2012) (DeBoer, et

445   al., 2014).

446   It has been shown that APP can be internalized from the cell surface and specifically targeted to

447   lysosomes(Tang, et al., 2015). Previous works demonstrated that the fluorescence emitted by

448   GFP can be strongly reduced by the acidic pH of the lysosomal lumen, while the fluorescence

449   emitted by mCherry remains stable in a broad range of pH (Katayama, et al., 2008;Mizushima,

450   Yoshimori and Levine, 2010). These considerations could in part explain the presence in our

451   images of intracellular vesicles with high red fluorescence intensity barely co-localizing with the

452   green fluorescence signal. However, the fact that we did not observe any co-localization with

453   the lysosome marker LAMP1 does not convincingly support this hypothesis. It is rather plausible

454   that the red punctae correspond to cleaved N-terminal fragments transported in vesicles

455   separated from those carrying the full length protein and the C-terminal fragments, as already

456   observed by Villegas et al. (2014).

457   As an alternative to the siRNAs, we have created a variant of mChAPPmGFP carrying a

458   mutation capable of inhibiting the α-secretase processing without altering the β-secretase

459   cleavage. The importance of this construct is the possibility to create transgenic animals, like

460   zebra-fish and rat, which could be used to produce a 3D map of the whole brain showing areas

461   with potentially different β-secretase activities. Previous data(Sisodia, 1992), together with our

462   confocal microscopy and FACS results, suggest that the P1 mutation makes APP cleavage-

463   resistant by α-secretase. However, it is unclear whether it completely inhibits the APP

464   processing. Although cells co-transfected with ADAM10mBFP and mChAPP^{P1}mGFP have a cell

465   number in the red/green area ratio similar to that of cells co-expressing mBFP and

466   mChAPPmGFP, the ratio for mBFP and mChAPP^{P1}mGFP transfected cells is much higher than

467   the ratio for ADAM10mBFP and mChAPP^{P1}mGFP transfected cells. It is possible that under

468   conditions where ADAM10 is strongly overexpressed, a small fraction of mChAPP^{P1}mGFP can

469   be still cleaved.

470   In general, FACS flow cytometry corroborated the reliability of our bioassay and confirmed the

471   heterogeneous processing of APP within the same type of cells. Furthermore, the possibility to

472   separate cells with different rates of APP processing, i.e. different red/green ratios, using the

473   cell-sorting capability of FACS might allow to perform a complete proteomic, lipidomic and

474   transcriptomic analysis of these sub-populations of cells, and to address one of the most

475   puzzling questions in the context of Alzheimer's disease: why the proteolytic processing of APP

476   is higher in certain cells than in others.

477   The combined approach presented in this work could be also used as versatile bioassay to test

478   in living cells the effect of specific molecules on the proteolysis of APP. A more detailed

479   knowledge of the factors governing the processing of APP could contribute to develop

480   alternative drugs for AD treatments.

481

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Figure 1

Schematic representation of the bioassay rationale.

(a) Design of the various chimeric constructs. The N-terminal tags are inserted after the signal peptide (SP) that target APP to the plasma membrane. HA and AP are two short amino acid sequences. HA is recognized by a specific antibody and AP is biotinylated by a specific enzyme. (b) Once mChAPPmGFP is cleaved by β-secretase, the sAPPβ fragment carrying mCherry (mCherry-sAPPβ) is released into the medium, causing a change in the ratio between the red and green fluorescence intensity.

a

b
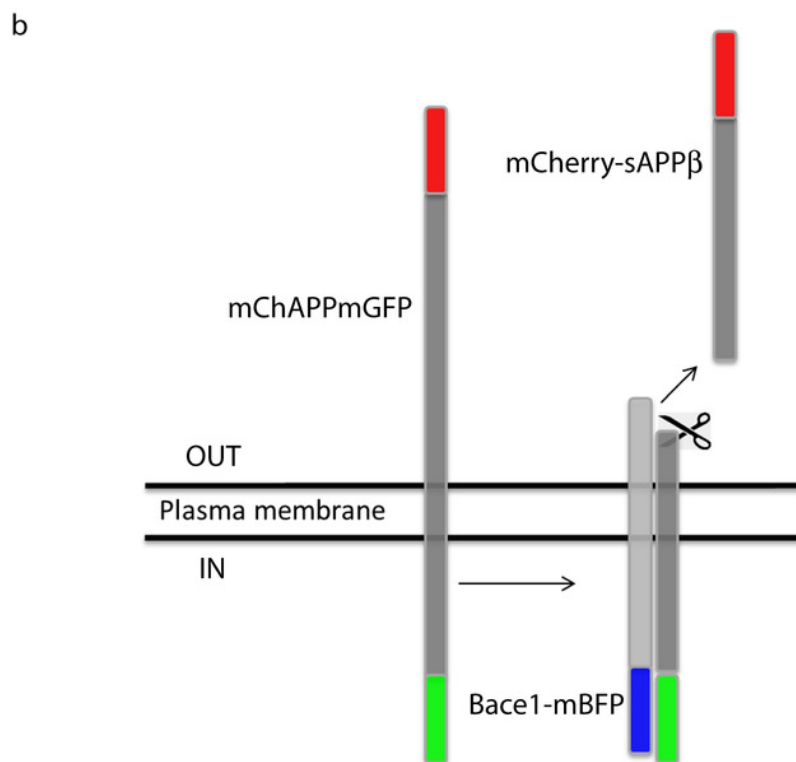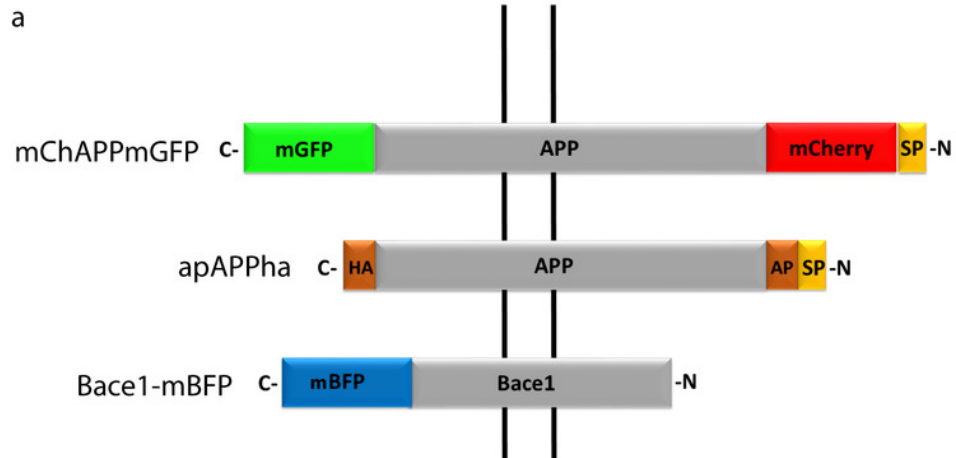
Figure 2

Correct targeting of mChAPPmGFP and colocalization of mCherry and mGFP.

(a) Maximum intensity projection of a confocal z-stack of fixed human SH-SY5Y cells transfected with mChAPPmGFP and surface labeled with anti-mCherry coupled to secondary Alexa 405 antibody (blue). Maximum intensity projections of confocal z-stacks of living human SH-SY5Y cells (b) and rat hippocampal neurons (e) transfected with mChAPPmGFP. (h) Maximum intensity projection of a confocal z-stack of fixed and permeabilized SH-SY5Y cells transfected with apAPPha and labeled with streptavidin Alexa 568 (red), which binds to the bionitylated AP tag, and anti-HA coupled to secondary Alexa 488 antibody (green). The high degree of co-localisation of the red and green signals evident from the linear correlation of scatterplots (c, f, i) and the exponential shape of the Li's intensity correlation analysis (d, g, j) is confirmed by the Pearson's (Pc) and Manders'coefficients (M1 and M2) close to 1, and by the intensity correlation quotients (ICQ) close to 0.5. The probability of obtaining the observed Pcs by chance is inversely correlated with the Costes' randomization P value, which is 100% in all cases. Scalebars, 10 μm.
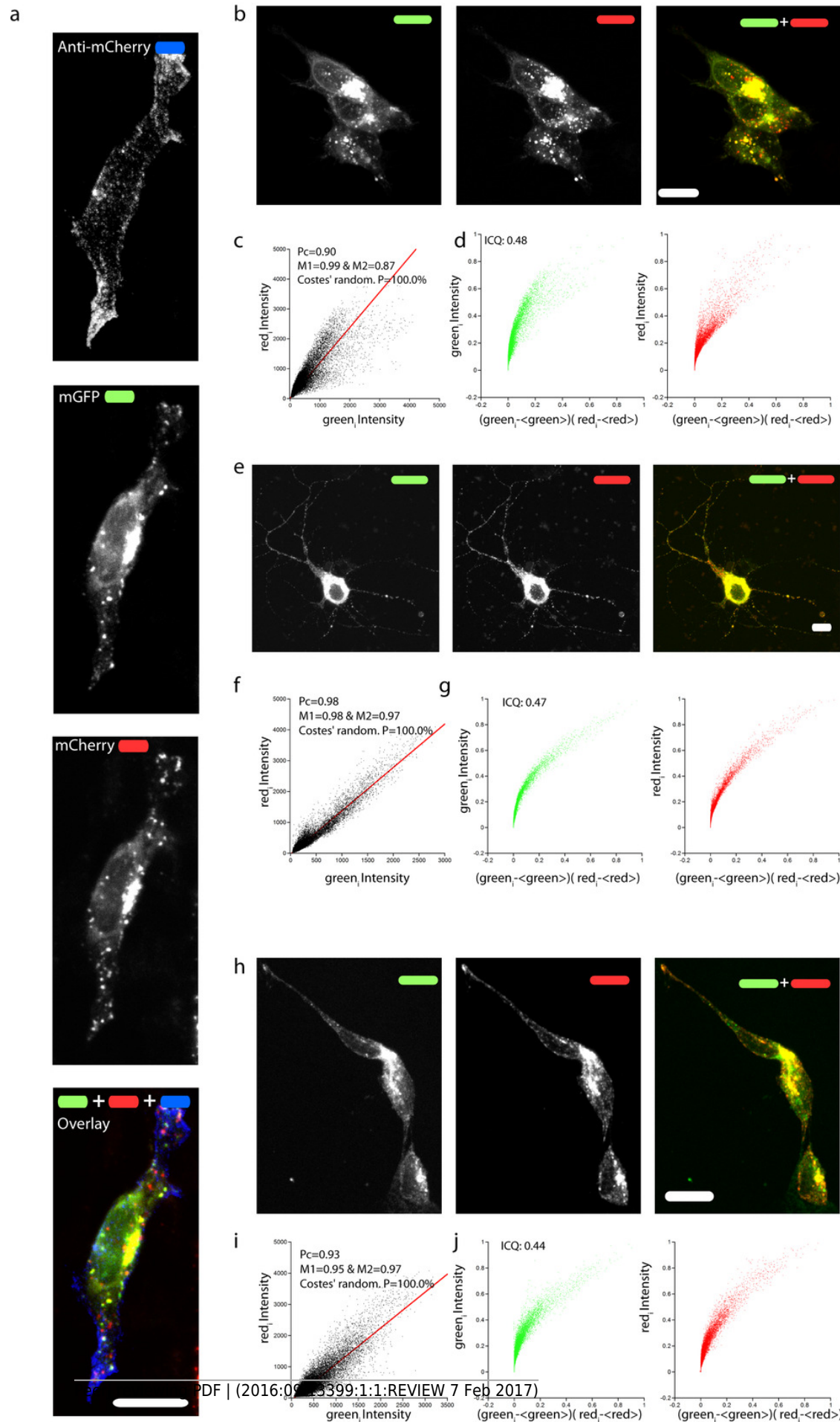
Figure 3

Red/green fluorescence intensity ratio and intercellular variability of mChAPPmGFP processing.

Maximum intensity projections of confocal z-stacks of living human SH-SY5Y cells (a) and rat hippocampal neurons (b) transfected with mChAPPmGFP. The average red/green fluorescence ratio can fluctuate within the same population of cells (examples indicated by coloured arrows). The red/green ratio image has been mean filtered (2 pixels) only for representation purposes. Scalebar 10 μm.
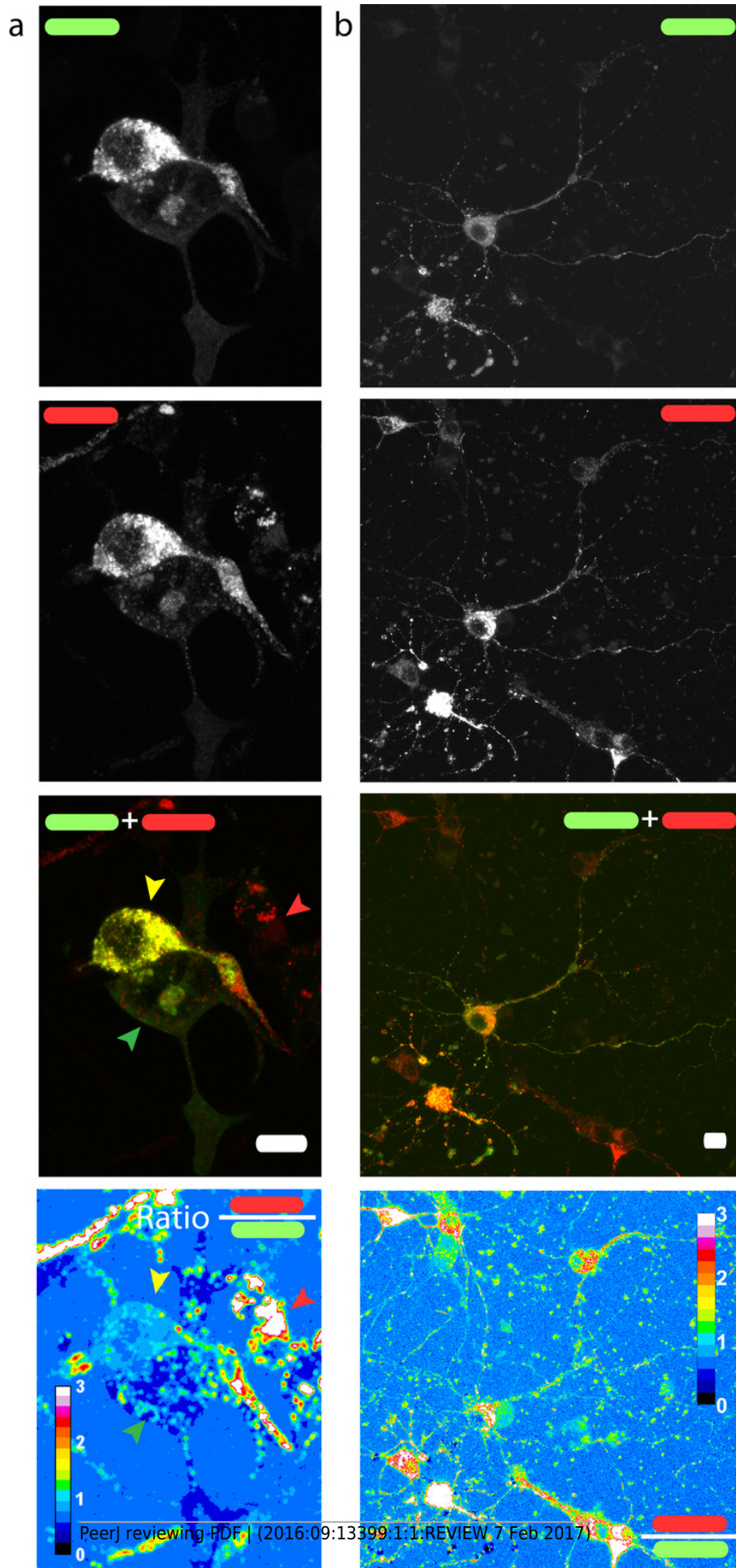
Figure 4

Proof of principle: overexpressed β-secretase cleaves efficiently mChAPPmGFP.

(a) Maximum intensity projection of a confocal z-stack of living human SH-SY5Y cells co-transfected with mChAPPmGFP and Bace1-mBFP. The red/green fluorescence ratio image has been mean filtered (2 pixels) only for representation purposes. Scalebar, 10 μm. (b) The different mean red/green fluorescence intensity ratio values of the two cells outlined in ratio image (a) are correlated to the overexpression of Bace1-mBFP (error bars, s.d.).(c) Dot blot of the mean red/green ratio of SH-SY5Y cells under different conditions of transfection. n > 15 for each condition; ***, p<0.001 according to unpaired Student-t test with unequal variance, run with respect to cells transfected only with mChAPPmGFP or mChAPP770mGFP. (d) Superimposed westernblots of transfected SH-SY5Y cell lysates (magenta) and extracellular media (cyan), labelled with anti-mCherry. The lysate blot was repeatedly stripped and reprobed with anti-GFP, anti-APP and anti-Aβ. The lower band corresponds to mCherry-sAPP, while the higher to full-length mChAPPmGFP. (e) The extracellular medium of SH-SY5Y cells under different conditions of transfection was analysed with a fluorescence spectrophotometer. Emission spectra were collected following excitation at 550 nm.
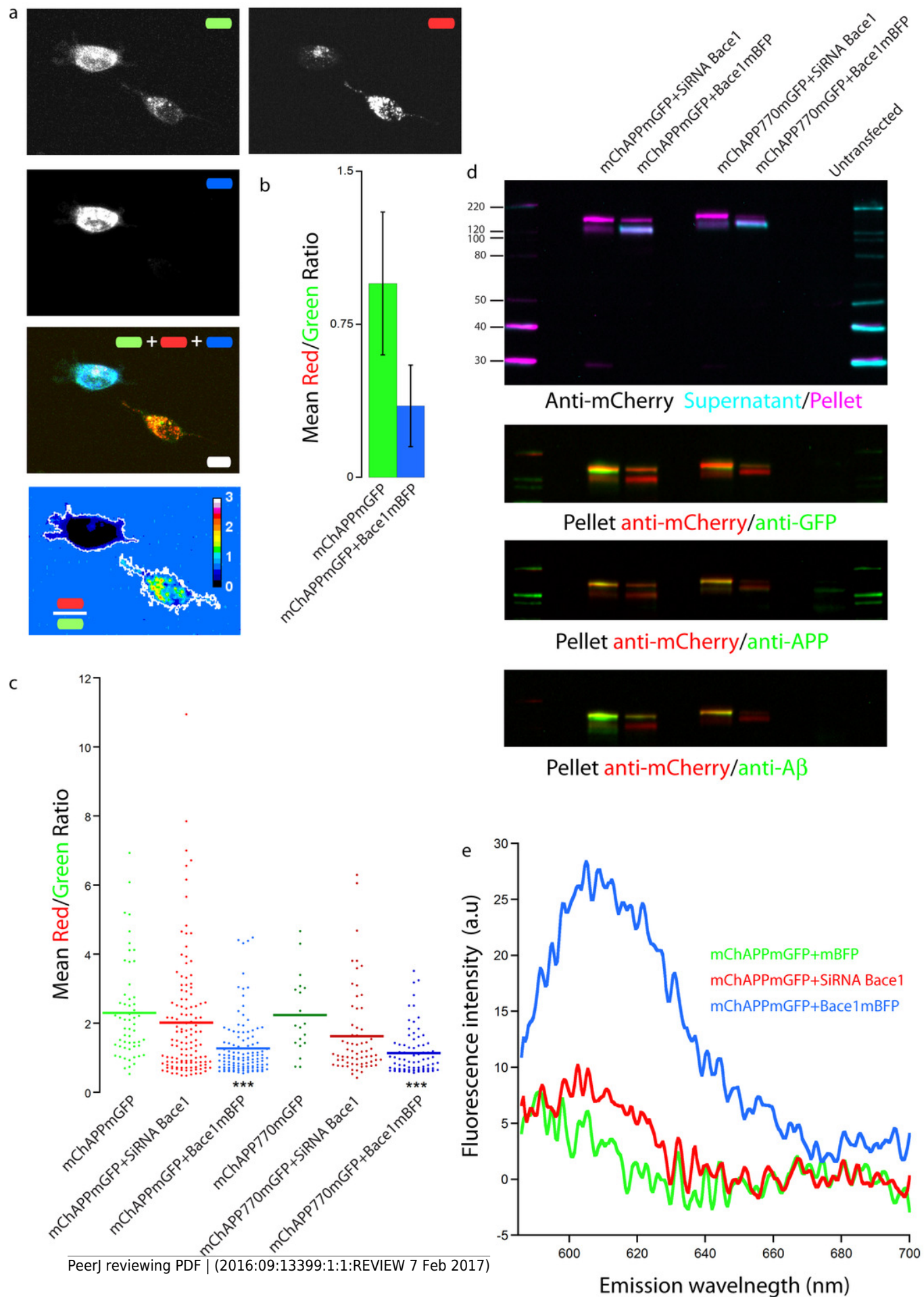
Figure 5

mChAPPmGFP is properly processed in neurons.

Maximum intensity projections of confocal z-stacks of living rat hippocampal neurons co-transfected with mChAPPmGFP and mBFP (a) or Bace1-mBFP (b). The red/green ratio images were mean filtered (2 pixels) only for representation purposes. The insets correspond to the magnified regions outlined in the ratio images. Scalebars 10 µm.
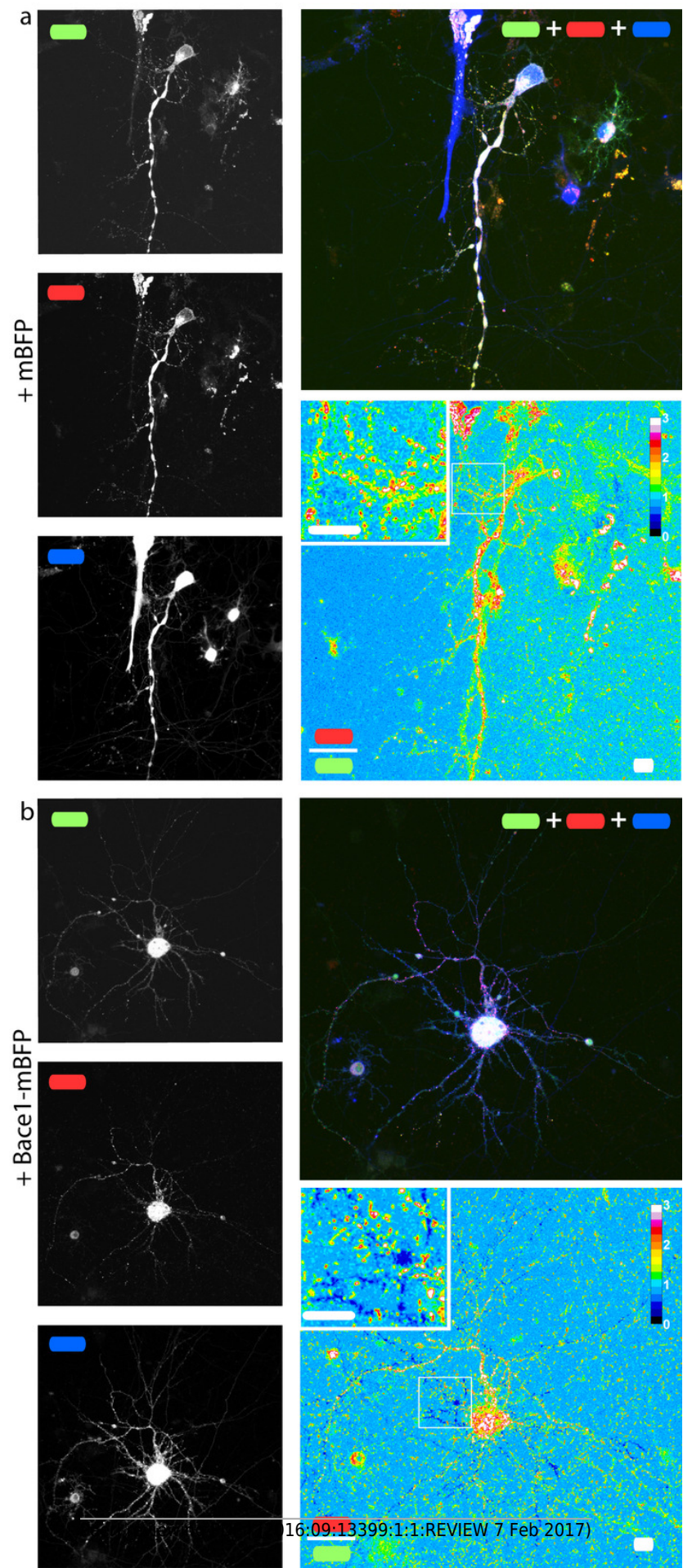
Figure 6

FACS flowcitometry as additional way to screen mChAPPmGFP processing.

(a) Each dot in the scatter plot represents the green and red fluorescence intensity of a single cell. The two different areas (red and green) in the scatter plot were arbitrarily chosen so that both areas contained the same number of SH-SY5Y cells co-transfected with mChaPPmGFP and control siRNA. These regions were used to gate the scatter plots of cells co-transfected with mChAPPmGFP and Bace1-mBFP, Bace1-siRNA, or control siRNA, and calculate the mean ratio between the number of cells in the red area over the number of cells in the green area (b). n = 3 independent experiments; error bars, s.d.; p<0.0001 according to unpaired Student-t test, run with respect to cells transfected with mChAPPmGFP and control siRNA.

a +siRNA contr

Log Red Fluorescence

Log Green Fluorescence

autofluorescent
non-transfected cells

+siRNA Bace1

Log Red Fluorescence

Log Green Fluorescence

+ Bace1

Log Red Fluorescence

Log Green Fluorescence

b

Ratio n cells Red/n cells Green

***

+siRNA contr   +siRNA Bace1   + Bace1
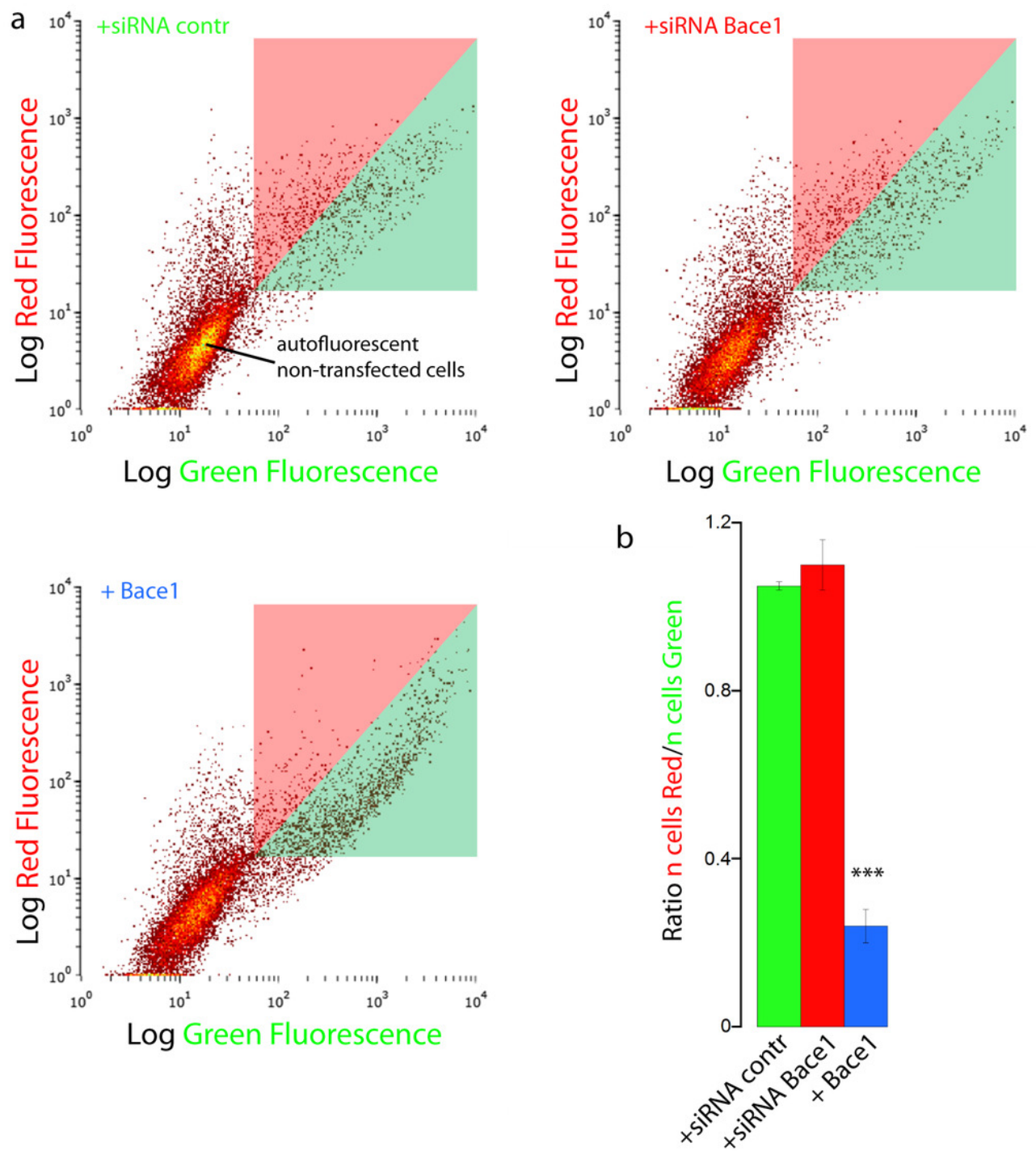
Figure 7

The mChAPP^{P1}mGFP mutant is not efficiently cleaved by the α-secretase ADAM10.

(a) Maximum intensity projection of confocal z-stacks of living human SH-SY5Y cells under different conditions of transfection. The red/green ratio images were mean filtered (2 pixels) only for representation purposes. Scalebar 10 μm. (b) Dot blot of the mean red/green ratio of SH-SY5Y cells under different conditions of transfection. n ≥ 10 for each condition; ***, $p<0.001$ according to unpaired Student-t test with unequal variance. (c) FACS flowcytometry confirms the inhibitory effect of the P1 mutation on α-secretase, but not β-secretase, processing. n = 3 independent experiments; error bars, s.d.; *, $p<0.05$, ***, $p<0.001$, according to unpaired Student-t test with unequal variance. Scatterplots from one experiment are reported in Supplementary Figure S7.