

1 **Amyloid- β induced membrane damage instigates tunnelling nanotubes and direct cell-**
2 **to-cell transfer**

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11
12 **Abstract:** The progressive pathology development in Alzheimer's disease is due to direct
13 transfer of amyloid- β_{1-42} oligomers (oA β) between connected neurons. However, the
14 mechanism of transfer is not revealed yet. Here we show that internalization of toxic oA β in
15 SH-SY5Y cells, causes cellular stress detected as significant membrane surface expansion.
16 Subsequently, protrusions appear in the form of tunnelling nanotubes (TNTs). The TNTs
17 propagate oA β and organelles directly from one cell-to-another. Preceding the formation, we
18 detect oA β induced plasma membrane damage and repair through lysosomal-exocytosis
19 followed by endocytosis to re-establish the membrane. Eventually, the non-degradable
20 internalized oligomers accumulate in lysosomes. Direct transfer of neurodegenerative proteins
21 via TNTs has recently been suggested as means of pathology spreading. However, molecular
22 basis of TNTs formation is unexplored. The present study is giving the insight that sprouting
23 of TNTs might instigate as consequences of oA β induced membrane damage and perturbed
24 membrane repair process in the stressed cells, probably to maintain cell surface expansion
25 and/or membrane-tension in equilibrium.

26
27 **Summary:** TNTs were recently discovered as novel route in cell-to-cell pathology spreading
28 of many diseases. Here we show that TNTs instigate as a consequences of perturbed membrane
29 repair process in oA β accumulated stressed cells. The mechanistic understanding has enormous
30 pathophysiology significance.

31
32 **Short title:** Direct cell-to-cell transfer of A β oligomers in TNTs.

33

34 **Key words:** Alzheimer's disease, Tunnelling nanotubes, amyloid- β , membrane repair,
35 endocytosis-exocytosis, prion propagation.

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41 **Introduction.** Neurodegenerative diseases are propagating disorders characterized by
42 accumulation of misfolded proteins that form aggregates, plaque and eventually cause
43 neurodegeneration. Prion like self-propagation and gradual pathology progression (Prusiner,
44 2013) in a predetermined pattern to different parts of the brain is a common hallmark of
45 neurodegenerative diseases. Several studies have shown that proteins involved in these
46 diseases such as tau, A β , α -synuclein and huntingtin follow common patterns including
47 misfolding, self-propagation and neuron-to-neuron transfer (Clavaguera *et al.*, 2009; Desplats
48 *et al.*, 2009; Ren *et al.*, 2009; Ilieva, Polymenidou and Cleveland, 2009; Hansen *et al.*, 2011).
49 In a model of Alzheimer's disease (AD), we have previously shown that spreading of AD
50 pathology is due to direct transfer of amyloidogenic oligomers of A β between connected
51 neurons (Nath *et al.*, 2012). Moreover, lysosomal stress due to gradual accumulation of toxic
52 non-degradable oA β enhances the progression and the cell-to-cell transfer correlates with
53 insufficient lysosomal degradation. Interestingly, transfer happens much before the cells start
54 to show detectable lysosomal toxicity (Domert *et al.*, 2014). The studies (Nath *et al.*, 2012;
55 Domert *et al.*, 2014) provided the possible explanations of how intracellular soluble oA β
56 reported as the potential initiator or driver of AD (Narasimhan *et al.*, 2017; Gouras *et al.*, 2010;
57 Walsh and Selkoe, 2004), could develop gradual pathology by propagating between connected
58 cells. However, mechanism of direct neuron-to-neuron propagation of neurodegenerative
59 aggregates is not revealed yet.

60 A β induced neuronal dysfunction and impaired synaptic plasticity accountable for memory
61 impairment reported to be propagated to the neighbouring cells (Kane *et al.*, 2000; Meyer-
62 Luehmann *et al.*, 2006; Wei *et al.*, 2010), however studies did not reveal if such pathology
63 propagation is due to direct trans-synaptic transfer of toxic A β . In addition, exosomes are
64 investigated as means of cell-to-cell transfer of A β (Rajendran *et al.*, 2006; Sardar Sinha *et al.*,
65 2018). However, these studies could not explain the anatomically connected strict
66 spatiotemporal pathology progression of AD. On the other hand, cell-to-cell transfers of both
67 extracellular and intracellular monomers and protofibrils of A β ₁₋₄₂ via TNTs are demonstrated

68 in the primary cultures of neurons and astrocytes (Wang et al., 2011). Recently, many studies
69 demonstrated TNTs to transfer neurodegenerative proteins, such as PrP^{Sc}, α -synuclein, A β , tau,
70 polyQ (Gousset *et al.*, 2009; Wang *et al.*, 2011; Zhu *et al.*, 2015; Tardivel *et al.*, 2016; Dieriks
71 *et al.*, 2017), directly from one cell-to-another. Several of these studies implicated links
72 between tunnelling nanotubes and endo-lysosomal pathway in cell-to-cell spreading (Bhat et
73 al., 2018; Victoria and Zurzolo, 2017). These studies and the discovery of TNTs (Rustom et
74 al., 2004) opened up new direction to prion like cell-to-cell propagation in neurodegenerative
75 diseases. However, the molecular basis of TNTs formation is unexplored.

76 TNTs are open ended membrane nano-structures with membrane actin protrusions between
77 neighbouring cells. The diameter of TNTs is reported to be between 50-200 nm (Gerdes et al.,
78 2013). TNTs are transient structures, stay intact for minutes to hours. Membrane protrusions
79 like filopodium precede TNT formation and inhibition of actin polymerization attenuates its
80 formation (Bukoreshtliev et al., 2009). TNT formation prevails in neuronal cells and primary
81 neurons (Rustom et al., 2004). Successive studies also showed TNT formation in different cell
82 types, such as immune cells, fibroblast, epithelial cells, astrocytes and many others (Onfelt *et*
83 *al.*, 2004; Davis and Sowinski, 2008; Gerdes and Carvalho, 2008).

84 In this study we show oA β induced formation of TNTs between neighbouring SH-SY5Y cells
85 and direct cell-to-cell propagation of oA β and organelles. Internalization of toxic oligomers of
86 oA β , causes cellular stress detected as significant membrane surface expansion. Subsequently,
87 TNTs protrude towards neighbouring cells. Preceding the formation of TNTs, we detect oA β
88 induced plasma membrane damage and repair through lysosomal exocytosis, and followed by
89 coupled-endocytosis to re-establish the plasma membrane. Eventually, the internalized and
90 transferred oligomers end up in lysosomes and non-degradable oligomers accumulates
91 gradually. Altogether, observations are giving insights that the mechanism of TNT formation
92 could be the consequences of perturbed membrane repair process in stressed cells. The study
93 is also revealing the involvement of oA β induced TNTs in direct neuron-to-neuron transfer of
94 amyloid pathology in AD progression.

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96

97 **Results.**

98

99 **oA β induce cellular stress and formation of TNTs.**

100 Cell-to-cell propagation of A β aggregates has enormous implication in Alzheimer's disease for
101 understanding the gradual progression of the pathology through anatomically connected brain

102 regions. Although cell-to-cell propagation of oA β between connected cells have been shown
103 in earlier studies (Wang *et al.*, 2011; Nath *et al.*, 2012; Domert *et al.*, 2014; Sardar Sinha *et al.*,
104 2018), however the mechanism is still unexplored. Here, we image the uptake of A β in
105 differentiated SH-SY5Y cells, after washing and staining with LysoTracker (green). We
106 observe that extracellularly applied oA β -TMR (10 nM to 1 μ M verified) get internalized
107 efficiently and significant amount is found to end up in lysosomes within 30 min. We have
108 quantified the internalization of 250 nM of oA β -TMR (red) after 3 h of incubation and observed
109 that 76 ± 14 % end up in lysosomes labelled with 200 nM of lysoTracker (green) by analysing
110 co-localization percentage (Figure 1A). Morphologically these cells exhibit blebs and
111 noticeable cell membrane expansion as well as nanotubes between neighbouring cells. Here,
112 we show images of cells incubated for 3 h with 250 and 500 nM of oA β -TMR (Supplementary
113 Movie 1, Figure 1B). Moreover, we show that oligomers of A β travel unidirectional from one
114 cell to another via TNTs (Figure 1B). The lengths of the TNTs are found to be between 0.2-16
115 μ m. We did not observe TNT structures and unusual membrane expansions in the differentiated
116 control cells (Figure 1C). Differentiated cells form neurites and in our previous studies we have
117 extensively characterized neuronal properties of those cells (Agholme *et al.*, 2010; Nath *et al.*,
118 2012). In the neurites, organelles and lysoTracker positive lysosomes move anterograde-
119 retrograde bi-directional (Supplementary Movie 2). We quantified the number of cells with
120 unusual blebs / lamellipodia and TNTs with respect to the total number of cells and found
121 concentration dependent increase, when quantifying at 200-500 nM of oA β -TMR treated cells
122 compare to the control cells (Figure 1D, E).

123

124 **oA β propagation to healthy cells is mediated by transport in TNTs.**

125 To study how cells with accumulated A β transfer the material to neighbouring healthy cells,
126 we have used a 3D donor-acceptor co-culture model that we earlier have designed (Nath *et al.*,
127 2012; Domert *et al.*, 2014). As acceptor cells, differentiated SH-SY5Y cells transfected with
128 EGFP-tagged endosomal protein (green, Rab5a) were used, while the donor cells were
129 incubated with oA β -TMR (red). Co-cultures were prepared by seeding donor and acceptor cells
130 in ECM gel on chambered glass slides and monitored in a confocal microscope. The images
131 captured by time laps recording show transfer of oA β -TMR donor (red) to acceptor cells
132 (green) (Figure 2A). Interestingly, acceptor cell that had internalized oA β -TMR forms TNTs
133 towards healthy differentiated acceptor cell. Thus, the oA β -TMR accumulated acceptor cells
134 form transient filopodium like structures and blebs, which are not detected in matured
135 differentiated neuronal controls. Noticeably, TNTs from stressed cells appear to grow from

136 bleb-like membrane protrusions (Figure 2A). Cells with blebs / lamellipodia and TNTs were
137 quantified by counting the images of acceptor cells transferred with oligomers and donor cells.
138 Acceptor cells having no transferred oA β -TMR, appear healthy as judged by appearance of
139 neurites and no significant blebs / lamellipodia (Figure 2B, C). DIC imaging using confocal
140 microscope is not an ideal method for capturing of nano-scale TNTs structures, especially in a
141 3D co-culture cellular model. Thus, it is a risk of under estimation in quantification.

142

143 **oA β induce formation of TNTs independent of neuronal differentiation.**

144 In order to clearly show that the studied TNTs structures are not neurites developed during
145 neuronal cell differentiation, we performed the experiments in partially differentiated cells and
146 undifferentiated SH-SY5Y neuronal cells. We have observed thin TNTs outspread from
147 expanded lamellipodia-like membrane protrusions in partially differentiated SH-SY5Y cells
148 internalized with oA β -TMR after incubation with 250 nM of oligomers for 3 h. Furthermore,
149 we detected unidirectional movement of oA β -TMR colocalized with lysoTracker labelled
150 lysosomes from one cell to another (Figure 3A). Similarly, we have observed TNTs outspread
151 from expanded lamellipodia and unidirectional movements of organelles (Figure 3B).
152 Moreover, the partially differentiated SH-SY5Y cells, form network of TNTs between
153 neighbouring cells (Figure 3C, D; Supplementary Movie 3). The cells make network between
154 3 neighbouring cells via TNTs, and those TNTs were also outspread from expanded
155 lamellipodia (Figure 3C). Formation of TNT networks between cells was also demonstrated in
156 the prevailing article, where they showed TNTs as mode of intercellular cell communication
157 (Rustom et al., 2004). We have also quantified oA β -TMR induced cells with unusual blebs /
158 lamellipodia and TNTs in partially differentiated cells treated with 250 nM of oA β -TMR for 3
159 h (Figure 3F), compare to the control cells (Figure 3E).

160 We have extended the experiments with undifferentiated SH-SY5Y cells. As presented in
161 Figure 4A, undifferentiated SH-SY5Y cells incubated with 1 μ M of oligomeric A β together
162 with the membrane dye TMA-DPH form TNTs within 10 min. Moreover, the oA β induced
163 TNT formation precedes the enhanced membrane activities, filopodium formation, blebs and
164 massive endocytosis process as compared to the control cells (Figure 4 A-C; Supplementary
165 Movie 4,5). oA β induced massive endocytosis was quantified by measuring the internalized
166 TMA-DPH from the luminal part of the cells (Figure 4C).

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170 **oA β ₁₋₄₂ induces membrane damage and lysosomal exocytosis.**

171 oA β induces TNT formation in association with the substantial enhancement of membrane
172 activities and massive endocytosis, similarly as evident in Ca²⁺ dependent repair of injured
173 plasma membrane (Idone et al., 2008), inspired us to determine if oA β has a role in membrane
174 damage. Previous reports have indicated that synthetic oA β makes ion-permeable pores in
175 synthetic membrane (Arispe, Pollard and Rojas, 1993; Kagan, 2012). A recent study has also
176 shown that oligomers of A β can induce a membrane repair response (Julien *et al.*, 2018).
177 Plasma membrane damage and influx of calcium has been shown to trigger lysosomal
178 exocytosis and formation of a patch over the damage membrane to prevent cell lysis. Likewise,
179 here we observe oA β (1 μ M) induced pore formation or membrane damage, detected as
180 appearance of Lamp1 on the extracellular leaflet of the plasma membrane, in undifferentiated
181 SH-SY5Y cells within 15-30 min of exposure (Figure 4E, F). To verify that the process is
182 calcium dependent, we analysed influx of the membrane-impermeant dye propidium iodide
183 (PI) in presence of 5 mM EGTA in PBS. The rationale behind this experiment is that if
184 lysosomal exocytosis-dependent plasma membrane repair is occurring, chelation of Ca²⁺ will
185 prevent the repair and PI will be detected intracellularly. As seen in Figure 4D, significant
186 enhancement of PI staining was detected within 30 min after exposure to oA β as quantified by
187 flow cytometry and confocal imaging. Thus, we conclude that addition of 1 μ M of oA β , cause
188 damage to the plasma membrane and as a membrane repair process lysosomal exocytosis and
189 fusion of lysosomal membrane with the plasma membrane occur. Subsequent to the membrane
190 repair process, re-establishment of the plasma membrane will occur by removing membrane
191 parts through endocytosis and as a consequence oA β internalizes into endosomes (Rab 5
192 positive structures) followed by entry into lysosomes (Lamp1 positive structures) (Figure 4G).
193 Eventually the accumulation of non-degradable oA β -TMR in lysosomes causes gradual
194 development of extra-large lysosomes (Figure 3H). We have reported in our earlier study
195 (Domert et al., 2014) that lysosomal stress develops by accumulated oligomers after 48 h.

196

197

198 **Discussion.** Prion like cell-to-cell propagation is a common characteristic of
199 neurodegenerative diseases. Several reports have persistently reported direct cell-to-cell
200 propagation of neurodegenerative protein aggregates and their implications in the gradual
201 pathology progression (Clavaguera *et al.*, 2009; Desplats *et al.*, 2009; Ren *et al.*, 2009; Ilieva,
202 Polymenidou and Cleveland, 2009; Hansen *et al.*, 2011). Our previous work has shown that, in
203 AD gradual pathology progression is due to direct transfer of intracellularly accumulated non-

204 degradable soluble oA β (Nath *et al.*, 2012; Domert *et al.*, 2014). However, the mechanism of
205 cell-to-cell propagation is unexplored. Synaptic transfer has been investigated as a potential
206 mechanism. A β induced spine loss and impaired synaptic plasticity are early pathologies and
207 develop before neurodegeneration (Wei *et al.*, 2010). Moreover, A β cause disruption of the
208 synaptic transmission mediated by the synaptic receptors NMDA and AMPA (Walsh and
209 Selkoe, 2004; Venkitaramani *et al.*, 2007). Therefore, neuron-to-neuron synaptic transmission
210 of oA β is unlikely. Likewise, inhibition of the possible receptors involved in synaptic
211 transmission did not show any effect on the cell-to-cell transfer of A β in the cultured
212 differentiated neuronal cells (Nath *et al.*, 2012).

213 A β peptides are mainly generated extracellularly from transmembrane amyloid precursor
214 protein (APP) by β - and γ -secretase enzymatic cleavage. Recent studies showed presence of β -
215 and γ -secretase machineries and APP at the membranes of endo-lysosomes and generation of
216 A β peptides in the lumen of endosomes and lysosomes (LaFerla, Green and Oddo, 2007;
217 Gouras *et al.*, 2010; Edgar *et al.*, 2015). Increasingly the clinical studies and animal models
218 indicate that soluble oA β is the disease initiator and driver, rather than large extracellular
219 depositions. Importantly, it was recently reported that the intracellular pool is mainly formed
220 from aggregating prone and toxic A β 42, while the less toxic A β 40 is more abundant
221 extracellularly (Sannerud *et al.*, 2016). Intracellular accumulation of amyloidogenic proteins
222 cause decreased lysosomal efficiency, detected as reduced degradation, abnormal lysosomal
223 morphology and lysosomal membrane permeabilization as hallmark of neurodegenerative
224 diseases (Freeman *et al.*, 2013; Eriksson *et al.*, 2017; Victoria and Zurzolo, 2017; Gowrishankar
225 *et al.*, 2015). Lysosomes are central hubs for nutrient homeostasis but is also actively involved
226 in cell death induction and plasma membrane repair. Stress signals from lysosomes also induce
227 various signals affecting mitochondria, nucleus and dysregulates various cellular processes,
228 and mediate increased oxidative stress (Appelqvist *et al.*, 2013; Ditaranto, Tekirian and Yang,
229 2001; Eriksson *et al.*, 2017; Freeman *et al.*, 2013). Several studies have indicated that ROS
230 induced cellular stress enhances TNT formation (Rustom, 2016). Notably, lysosomal
231 stress/damage due to accumulation of non-degradable amyloidogenic aggregates could induce
232 formation of TNTs (Abounit *et al.*, 2016) and cell-to-cell transfer of oA β correlates with
233 insufficient degradation efficiency of lysosomes (Domert *et al.*, 2014).

234 In contrast, impaired processing of A β due to lysosomal enzymatic inefficiency and enhanced
235 exocytosis was demonstrated by Annunziata *et al.*, 2013. Additionally, the study by Hase *et*
236 *al.* 2009 also reported that the exocyst complex involved in exosome fusion and membrane
237 expansion regulates formation of TNTs. Plasma membrane recruitment of Ral-GTPase and

238 filamin, the actin remodeling proteins, also indicate positive regulating effects in TNT
239 formation (Hase et al., 2009). The present study also demonstrates unusual membrane
240 expansion, blebs, filopodium-like structures and outspread of TNTs from expanded
241 membranes. Upregulation of exocyst complex during TNT formation might be prearrangement
242 of rapid exocytosis as a Ca^{2+} -dependent membrane repair process. The observation of active
243 generation of Ca^{2+} and its propagation through TNTs has already been reported by Smith et al.,
244 2011. Recently, it was also shown that oligomers of A β can induce a membrane repair response
245 similar to that induced by exposure to the bacterial pore-forming toxin produced by *B.*
246 *thuringensis* (Julien et al., 2018). Consequently, enhanced internalization of A β occur via
247 endocytosis, which is independent of receptor interactions.

248 Internalization of the oA β pool appears to occur via early endosomes and is directed to
249 lysosomes, where non-degradable aggregation prone oA β gradually induce lysosomal stress.
250 Notably, propagation of amyloidogenic seeds was observed much earlier, whereas lysosomal
251 pathology develops gradually over time (Domert et al., 2014). Involvement of the endo-
252 lysosomal pathway is also relevant in maintaining cellular homeostasis by regulating
253 membrane stress and cell surface expansion (Apodaca, 2017; Thottacherry et al., 2018).
254 Exocytosis is involved in the expansion of cell surface area and results in decreased membrane
255 stress. Simultaneously, the process of endocytosis follows to re-establish the membrane area
256 or to re-establish the membrane stress. Recent studies have shown the formation of tubular-like
257 filopodium structures, membrane invaginations/blebs during induction of reduced cellular
258 stress (Norman et al., 2010; Sinha et al., 2011; Kosmalska et al., 2015). Similarly, the TNTs
259 form from protrusions in response to rapid membrane repair could be the consequences of
260 perturbed membrane repair process in cells stress by accumulated oligomers, probably to
261 maintain the membrane stress and cell surface expansion in equilibrium.

262 Several studies have suggested exosomes as means of cell-to-cell transfer of A β (Rajendran et
263 al., 2006; Sardar Sinha et al., 2018). However, such experiments were designed at non-
264 physiological conditions, with purified concentrated exosomes from large number of cultured
265 cells. Additionally, a transfer mechanism via exosomes is unable to explain the asymmetric
266 spreading of oA β microinjected in a single neuron of primary hippocampal culture (Nath et al.,
267 2012). Studies using the method of transwell assays have also shown the efficient transfer of
268 prion proteins between cell-to-cell despite the blocking of exosomes (Gousset et al., 2009;
269 Thayanithy et al., 2017). The studies indicated the possible mechanism of direct cell-to-cell
270 transfer via TNTs. On the other hand, organelles like toxic lysosomes have been demonstrated
271 to propagate directly cell-to-cell through TNTs (Victoria and Zurzolo, 2017). Moreover,

272 increasingly reports of cell-to-cell transfer of common neurodegenerative proteins via TNTs,
273 such as PrP^{Sc}, α -synuclein, tau, polyQ aggregates and A β , instigate new avenues (Gousset *et*
274 *al.*, 2009; Wang *et al.*, 2011; Zhu *et al.*, 2015; Tardivel *et al.*, 2016; Dieriks *et al.*, 2017). TNTs
275 as means of direct neuron-to-neuron transfer is a convincing model of how the oligomers
276 suggested as initiator or driver of AD pathology could gradually progress through the
277 anatomically connected brain regions. The hypothesis is important in the context of progressive
278 AD pathology development, as plaque pathology poorly correlates with progressive cognitive
279 impairment in AD. Here, our study gives insight that the formation of TNTs could be the
280 consequences of perturbed equilibrium balance between coupled endocytosis-exocytosis
281 during rapid membrane repair (Figure 5). However, further in-depth studies are needed to
282 understand how cells maintain homeostasis of intercellular communication by balancing
283 exosome release and TNTs in the stressed cells. It is also important to unfold the involvement
284 of cell surface expansion and endo-lysosomal pathway in the induction of TNTs formation at
285 molecular level.

286 Unfolding of molecular basis of TNT formation is immensely demanding, as it is an important
287 intercellular communication mechanism of stressed cells reported in many diseases like
288 neurodegenerative diseases, HIV-1 infection (Sowinski *et al.*, 2008; Hashimoto *et al.*, 2016),
289 Influenza virus (Kumar *et al.*, 2017), Herpesvirus transmission (Panasiuk *et al.*, 2018) and
290 cancer (Lou *et al.*, 2012). Recent, lattice light sheet imaging directly visualized membrane
291 nanotubes *in situ*, in interconnecting breast cancer cells in live acute brain slices (Parker *et*
292 *al.*, 2017). Thus, the understanding the mechanism of TNT formation has huge
293 pathophysiological significance. The study will open up a new avenue to unfold and explore an
294 emerging novel pathway of intercellular cell-to-cell communication.

295

296

297 **Material and Methods.**

298

299 **Preparation of soluble oA β .** Freshly made unlabelled oA β and fluorescently labelled oA β -
300 TMR were prepared from lyophilized A β (A β ₁₋₄₂) and A β -TMR (A β ₁₋₄₂-5-tetramethyl
301 rhodamin) suspended in 1,1,1,3,3,3-hexafluoro-2-propanol (AnaSpec). Lyophilized A β and
302 A β -TMR were resuspended at a concentration of 5 mM in Me2SO and then diluted to a
303 concentration of 100 μ M in HEPES buffer, pH 7.4. The solution was immediately vortexed
304 and sonicated for 2 min and then incubated at 4°C for 24 h (Catalano *et al.*, 2006; Nath *et al.*;
305 Domert *et al.*). Oligomers were characterized before doing the experiments similarly as reported

306 in our earlier papers (Nath *et al.*, 2012; Domert *et al.*, 2014), by electron microscopy imaging
307 using Jeol 1230 transmission electron microscope equipped with an ORIUS SC 1000 CCD
308 camera, together with SDS-PAGE, Native-PAGE western blots and size exclusion
309 chromatography.

310

311 **Cell culture and differentiation of cells.** SH-SY5Y neuronal cells (ECACC; Sigma-Aldrich)
312 were seeded on 10-mm glass-bottom Petri dishes (MatTek) at a concentration of 12,000
313 cells/cm². Cells were partially differentiated with 10 μ M retinoic acid (RA; Sigma-Aldrich) for
314 7 days. Pre-differentiated or partially differentiated SH-SY5Y cells were further differentiated
315 for additional 10 days in 10-mm glass-bottom Petri dishes (MatTek) with brain-derived
316 neurotrophic factor, neuregulin-1, nerve growth factor, and vitamin D3. After 10 days of
317 differentiation on glass, the cells form long, branched neurites and several neurospecific
318 markers, as described previously (Agholme *et al.*, 2010; Nath *et al.*, 2012). Partially
319 differentiated and differentiated cells on glass petri dishes were incubated with 100–500 nM
320 oA β -TMR for 3 h at 37°C in a 5% CO₂ atmosphere. The cells were imaged after extensive
321 PBS washing (two washes of 10 min each at 37°C with 5% CO₂). LysoTracker (green;
322 Invitrogen) 50-250 nM were added 5-10 min before imaging.

323

324 **Donor-acceptor 3D co-culture.** Three dimensional donor-acceptor co-culturing was
325 performed using RA treated partially differentiated cells for 10 days in ECM gel (1:1; Sigma-
326 Aldrich), as described earlier (Nath *et al.*, 2012; Domert *et al.*, 2014). The differentiated cells
327 in ECM gel have been well characterized previously; they had long, branched neurites, several
328 neurospecific markers, synapse protein Sv2, axonal vesicle transport and mature splicing forms
329 of tau (Agholme *et al.*, 2010; Nath *et al.*, 2012). The differentiated cells were stained green
330 with EGFP-tagged (green) endosomal (Rab5a) or lysosomal (Lamp1) proteins using organelle
331 lights BacMam-1.0 or 2.0 (Invitrogen). The green labelled differentiated cells were called
332 ‘acceptor cells’. Pre-differentiated cells (10 μ M RA for 7 days in 35-mm cell-culture Petri
333 dishes) incubated with 500 nM oA β -TMR for 3 h at 37°C in a 5% CO₂ atmosphere; extensively
334 washed using PBS (two washes of 10 min each at 37°C with 5% CO₂) and trypsinized, were
335 denoted as ‘donor cells’ reseeded (40,000 cells per dish) on top of the acceptor cells after
336 mixing (1:1) with prechilled ECM gel. Then, the 3D donor-acceptor co-cultured cells were
337 incubated for 24 h with 10 μ M RA at 37°C in a 5% CO₂ atmosphere.

338

339 **Immunocytochemistry.** Lamp1 staining on the luminal part of the plasma membrane in
340 undifferentiated SH-SY5Y neuroblastoma cells was performed similarly as described earlier
341 (Wäster et al., 2016), on unfixed cells. Cells were incubated for 15 and 30 min with 1 μ M of
342 oA β in MEM media without FCS (fetal calf serum) supplement. Then endocytosis of the cells
343 were blocked with 5% BSA + 10% FCS in PBS for 5 min at 4 °C. Then cells were incubated
344 with Lamp1-anti goat primary antibody (1:250, sc-8099, Santa Cruz Bio-technology; Santa
345 Cruz, CA, USA; 2 h, 4 °C) in the blocking buffer for 45 min, followed by fixation of the cells
346 in 4% PFA for 20 min at 4 °C before labelling with the secondary anti-goat antibody conjugated
347 to Alexa Fluor 488 (1:400 for 30 min; Molecular Probes, Eugene, OR, USA). Next, the cells
348 were mounted in ProLong Gold antifade reagent supplemented with 4',6-diamidino-2-
349 phenylindole (DAPI; Molecular Probes).

350

351 **Propidium Iodide staining:** Undifferentiated SH-SY5Y cells were incubated with 1 μ M of
352 oA β for 15 or 30 min at 37 °C. Then the cells were washed and stained with propidium iodide
353 (PI, 5 μ g/ml) by incubating for 5 min. Cells were washed again two times with PBS before
354 applying 4 % PFA as fixative for 20 min at 4 °C. The fixed cells were observed by confocal
355 microscopy or trypsinized before analyzing by flow cytometer.

356

357 **Live cell imaging of membrane dynamics:** oA β of concentration 1 μ M was added
358 concurrently with 0.5 μ M of membrane binding dye TMA-DPH (N,N,N-Trimethyl-4-(6-
359 phenyl-1,3,5-hexatrien-1-yl) phenylammonium p-toluenesulfonate; molecular formula:
360 C₂₈H₃₁NO₃S) and membrane dynamics of the live cells were followed by timelaps images
361 using a confocal microscope. The excitation and emission maximum of TMA-DPH is 384 and
362 430 nm, with considerable tail of excitation spectra at 405 nm. Therefore, time-lapse images
363 were taken using confocal-microscope by exciting the TMA-DPH dye using the 405 nm laser
364 and sufficient emission light was collected using an opened pinhole. In this setup confocal
365 microscope produces images similar to the widefield or epifluorescence images. Cultures were
366 carried to the microscope one by one in 500 μ l of 20 mM HEPES buffer of pH 7.4 maintaining
367 the temperature at 37 °C.

368

369 **Confocal microscopy and flow-cytometry.** All images were acquired using a Zeiss LSM laser
370 scanning confocal microscope using 63x/1.40 NA or 40X/1.3 NA oil immersion plan-
371 apochromatic objective (Carl Zeiss AG, Oberkochen, Germany). The time-laps image
372 sequences of the live cells were taken at 37 °C by capturing simultaneously differential

373 interference contrast (DIC) and fluorescence modes. Cells internalized with propidium iodide
374 (PI) were fixed, trypsinized and filtered using CellTrics 30 μm filters (Sysmex). Then re-
375 suspended in PBS and quantified using BD FACS AriaTM (BD Biosciences) flow cytometer.

376

377 **Image analysis.** Image analysis were done by ImageJ software (open source by NIH, USA).
378 Percentage of co-localization of $\text{oA}\beta$ (red) with lysosomes (green) were performed by
379 calculating the proportion of the red fluorescence pixels compared to the co-localized pixels
380 from background subtracted images using Coloc-2 plugin. Cells with blebs / lamillopodia and
381 TNTs were counted from images and normalized with total number of cells. $\text{oA}\beta$ induced
382 endocytosis was quantified by measuring the integrated intensities of internalized TMA-DPH
383 from the luminal part of each cells by drawing ROI (region of interest) over sequences of time-
384 laps confocal image stacks and compared the same quantification with control cells. The fusion
385 of lysosomal membrane to reseal the membrane damaged was detected as appearance of
386 LAMP1 (green) on the outer leaflet of the plasma membrane. The outer leaflet LAMP1 was
387 quantified measuring the integrated intensities from drawn ROI and proportionate percentage
388 calculated comparing the total LAMP1 per cells.

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390

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399

400

401 **Author Contributions:** : S.N conceptualized and conducted the research; S.N and K.O
402 designed research; S.N designed tunnelling nanotube experiments; K.O. designed membrane
403 dynamics and membrane repair experiments; S.N performed experiments; S.N, K.O and K.K
404 interpreted data; S.N wrote the paper taking valuable inputs from K.O and K.K.

405

406

407 **Abbreviations.**

408 AD - Alzheimer's disease.

409 oA β - Oligomers of amyloid- β (1-42)

410 oA β -TMR – Tetramethyl rhodamin labelled oligomers of amyloid- β (1-42)

411 TNTs- Tunnelling nanotubes

412 LAMP-1 - Lysosomal associated membrane protein-1

413

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610 **Figure Legends**

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612 **Figure 1.** A) Differentiated SH-SY5Y cells were incubated with 250 nM of oA β -TMR (red)
613 for 3 h, washed and imaged by labelling with LysoTracker (200 nM; green). oA β -TMR ends
614 up in lysosomes labelled with lysotracker. B) Connected nanotubes (highlighted by rectangle
615 boxes) between neighbouring cells detected in differentiated SH-SY5Y cells incubated for 3 h
616 with 500 nM of oA β -TMR (red), washed and stained with 50 nM of lysotracker (green) before
617 taking the image. The cells with long TNTs form noticeable blebs, filopodium and cell
618 membrane expansion (indicated by black arrows). Unidirectional movements of oA β -TMR
619 were observed, indicated by white arrows in the sequence of images. C) Differentiated control
620 cells show neuritic connections. The cells are also devoid of TNTs and unusual blebs or
621 filopodium like structures. D) Percent of cells with blebs / lamellipodia were quantified from
622 the images taken with increasing concentrations of oA β -TMR (200-500 nM) and compared
623 with the control cells. E) Similarly the number of TNTs were quantified with respect to the
624 total number of cells. Quantifications were done from > 60 cells in each sets. Plots are mean \pm
625 SD. One-way ANOVA test were performed to validate statistical significance. Scale bars are
626 10 μ m.

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628

629 **Figure 2.** The 3D donor-acceptor cell model was desinged by donor cells allowed to
630 accumulate oA β -TMR (red) before co-cultured for 24 h with acceptor cells transfected with
631 EGFP-tagged (green) Lamp1. A) TNT formed towards a healthy acceptor cell (marked 2) from
632 the acceptor cell (marked 1) that already accumulated oA β -TMR. Box 1 shows enlargement of
633 transient filopodium (black arrows). Box 2 shows TNT formation from expanded membrane
634 protrusions, bleb like structures (white arrow). B) Percent of cells with blebs / lamellipodia in
635 oA β -TMR accumulated donor and acceptor cells were quantified by counting images after 24
636 h of co-culture and compare with the healthy acceptor cells with no accumulation of oA β -TMR.
637 C) Number of TNTs were also quantified with respect to the total number of donor and acceptor
638 cells, respectively. Quantifications were done from > 30 cells in each sets. Plots are mean \pm
639 SD. One-way ANOVA test were performed to validate statistical significance. Scale bar is 10
640 μ m.

641

642 **Figure 3.** Partially differentiated SH-SY5Y cells were incubated with 250 nM of oA β -TMR
643 (red) for 3 h, washed and labelled with 200 nM of lysotracker (green). Cells internalized oA β -
644 TMR and the oligomers accumulate to lysosomes. A) The cells form thin TNTs outspread from
645 expanded lamellipodia like membrane protrusions (black arrows). oA β -TMR colocalized with
646 a lysosome moves unidirectional away from one cell to another (white arrows). B) TNTs
647 outspread from expanded lamellipodia-like membrane protrusions (black arrows) and
648 unidirectional movements of an organell (white arrows). C) Network between 3 neighbouring
649 cells via formation of TNTs (white arrows), outspread from lamellipodia-like membrane
650 protrusions (black arrows). D) TNTs form networks between two neighbouring cells (black
651 arrow) (Supplementary Movie 3). E) Partially differentiated control cells devoid of blebs /
652 lamellipodia. F) Percent of cells with blebs / lamellipodia in oA β -TMR treated cells were
653 quantified and compared with the control cells. Number of TNTs were also quantified by
654 manually counting from cell images and plotted with respect to the total number of cells in
655 percentage. Plots are mean \pm SD. Quantifications were done from > 60 cells in each sets. One-
656 way ANOVA test were performed to validate statistical significance. Scale bars are 10 μ m.

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659 **Figure 4.** A) Undifferentiated SH-SY5Y cells incubated with 1 μ M of oA β together with the
660 membrane dye TMA-DPH (0.5 μ M) and imaged immediately after addition. TNT formation
661 (white arrows) were observed (Box 1 and 2) within 10 min, preceding of massive membrane
662 activities, filopodium formation and endocytosis of membranes. B) Control cells show no
663 blebs, filopodium or TNT structures. C) Internalization or endocytosis of plasma membranes
664 labelled with TMA-DPH, were quantified in control and oA β treated cells by measuring
665 luminal part of intensities over time. The plot is mean intensities with SD (quantified > 10 cells
666 from each set, n = 3). D) oA β (1 μ M) induced membrane damage in undifferentiated SH-SY5Y
667 cells, detected as uptake of the membrane-impermeable dye propidium iodide (PI) in presence
668 of 5 mM EGTA in PBS. Significant enhancement of PI uptake within 30 min of oA β treatment
669 was observed in confocal images and quantified by flowcytometry (n = 6). E) Enhanced Lamp1
670 surface staining was detected in undifferentiated SH-SY5Y cells within 30 min of exposure of
671 oA β (1 μ M), as an indicator of lysosomal membrane fusion with the plasma membrane as
672 mechanism of membrane repair process. F) Lamp1 surface staining was quantified from
673 intensity (plotted mean \pm SD) measurements by defined ROI in the image J. (number of cells
674 n for; control = 51, A β _15 min = 42 and A β _30 min = 6). Mann-Whitney t-test was performed

675 to validate the significance. G) Undifferentiated SH-SY5Y cells incubated with extracellularly
676 applied oA β (1 μ M) internalize to early-endosomes (Rab 5) and lysosomes (Lamp1) in
677 substantial quantity within 30 min. Here, the images are after 1.5 h of incubation. H) The
678 transferred non-degradable oA β -TMR (red) end up to lysosomes (Lamp 1, green) of acceptor
679 cells in the co-cultured cells. However, gradual accumulation and formation of extra-large
680 lysosomes was observed about 48 h. Scale bars are 10 μ m.

681

682

683 **Figure 5.** Schematic summary to show the involvement of oA β induced membrane damage
684 and membrane repair process in direct cell-to-cell transfer of oligomers via TNTs. Exocytosis
685 is followed by fusion of lysosomal membrane to reseal the damaged membrane on the outer
686 leaflet of the plasma membrane. Then, the repair process is finalized by endocytosis to re-
687 establish the plasma membrane.

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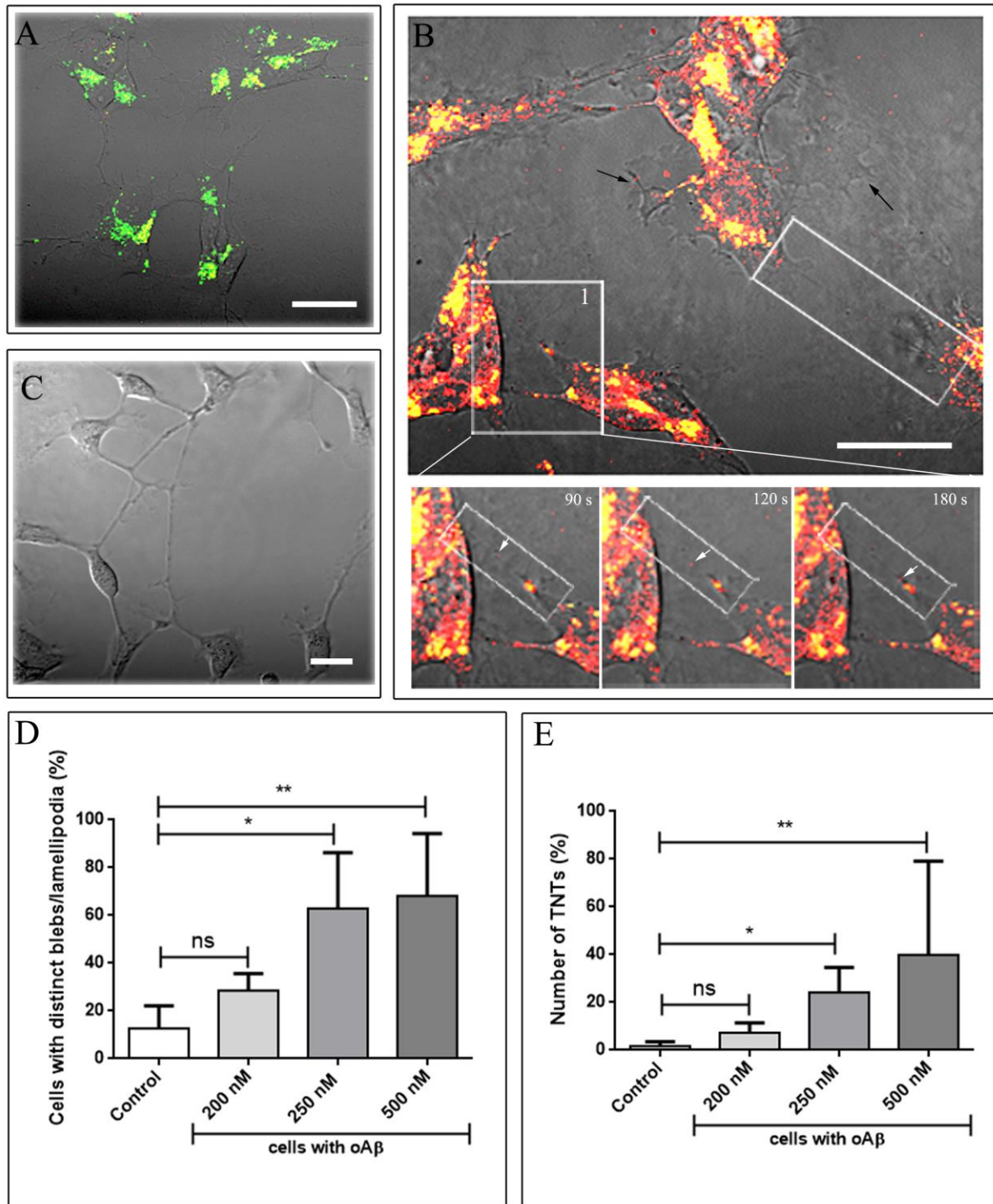
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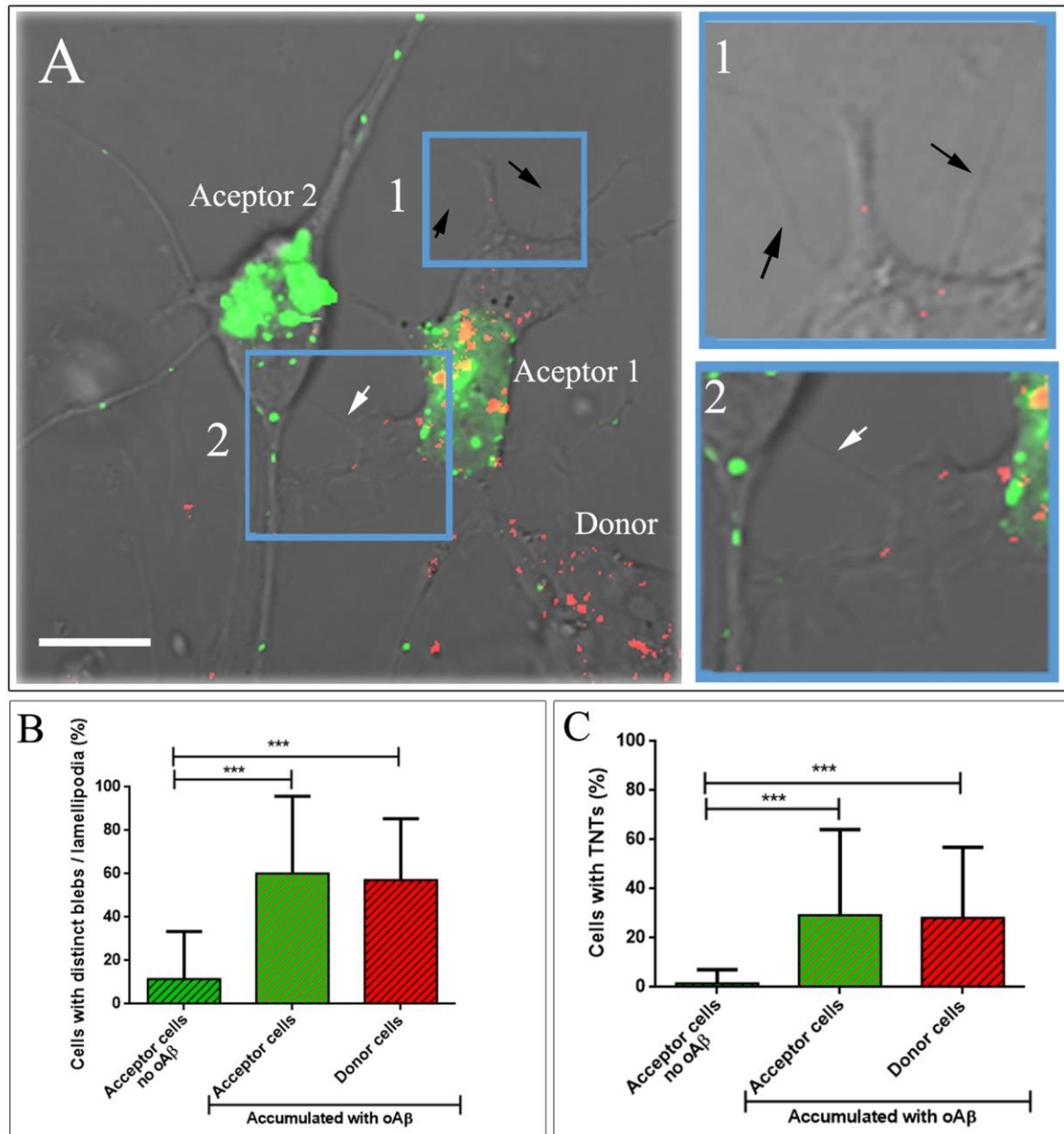
710 **Figure 1**

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716 **Figure 2**

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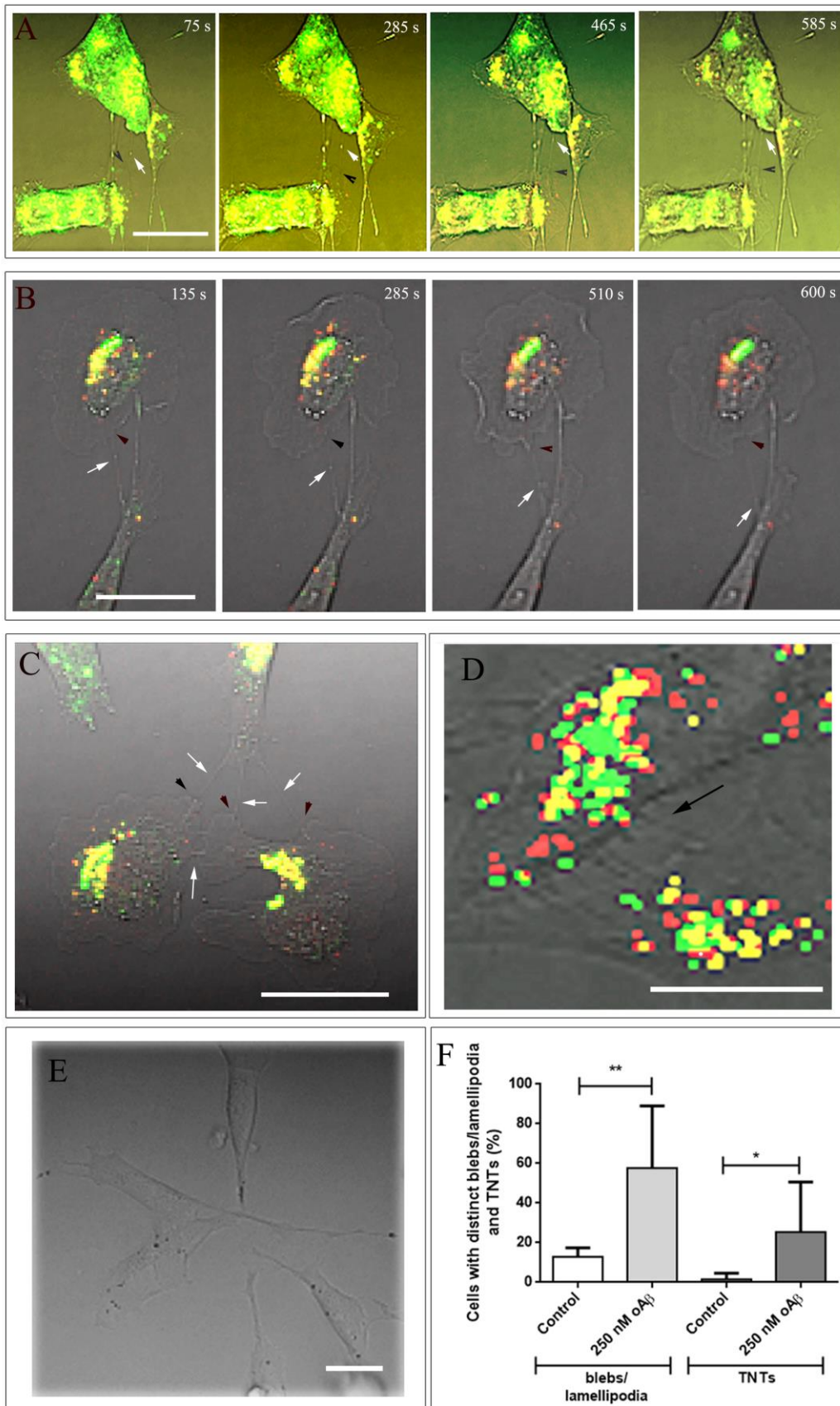
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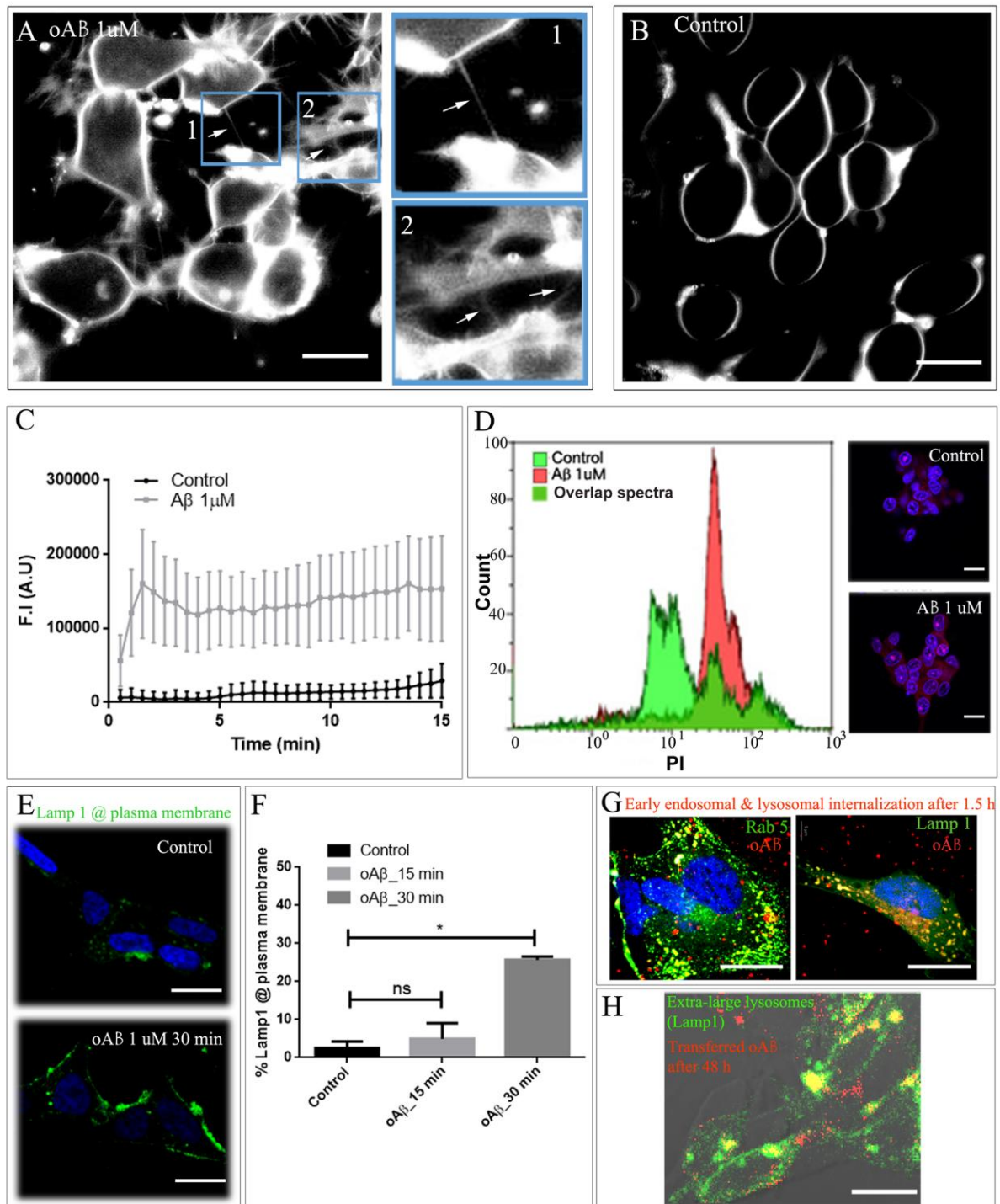


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727 **Figure 3**

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731 **Figure 4**

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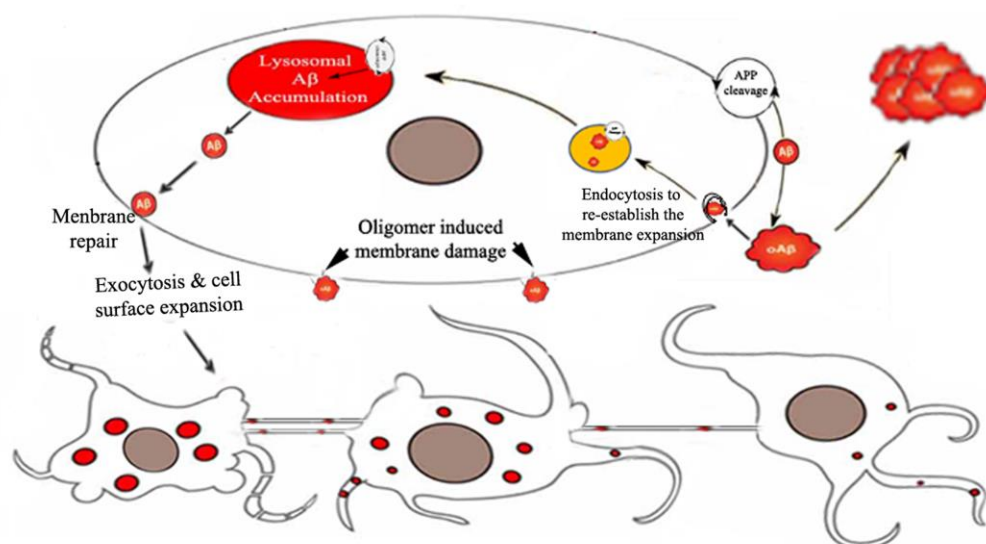
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739 **Figure 5**

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