Metadata of the Book that will be visualized online

Book Title	Amyloid Proteins	
Book SubTitle	Methods and Protocols	
Copyright Year	2012	
Copyright Holder	Springer Science+Business Media, LLC	
Corresponding Author	Family Name	Villalobos
	Particle	
	Given Name	Carlos
	Suffix	
	Division	
	Organization	Instituto de Biología y Genética Molecular (IBGM), Universidad de Valladolid and Consejo Superior de Investigaciones Científicas (CSIC)
	Address	Valladolid, Spain
	Email	carlosv@ibgm.uva.es
Author	Family Name	Caballero
	Particle	
	Given Name	Erica
	Suffix	
	Division	
	Organization	
	Address	
	Email	
Author	Family Name	Sanz-Blasco
	Particle	
	Given Name	Sara
	Suffix	
	Division	
	Organization	
	Address	
	Email	
Author	Family Name	Núñez
	Particle	
	Given Name	Lucía
	Suffix	
	Division	
	Organization	
	Address	
	Email	

Study of Neurotoxic Intracellular Calcium Signalling 2
Triggered by Amyloids 3

Carlos Villalobos, Erica Caballero, Sara Sanz-Blasco, 4
and Lucía Núñez 5

Abstract 6

Neurotoxicity in Alzheimer's disease (AD) is associated to dishomeostasis of intracellular Ca^{2+} induced by amyloid β peptide ($\text{A}\beta$) species. Understanding of the effects of $\text{A}\beta$ on intracellular Ca^{2+} homeostasis requires preparation of the different $\text{A}\beta$ assemblies including oligomers and fibrils and the testing of their effects on cytosolic and mitochondrial Ca^{2+} in neurons. Procedures for cerebellar granule cell culture, preparation of $\text{A}\beta$ species as well as fluorescence and bioluminescence imaging of cytosolic and mitochondrial Ca^{2+} in neurons are described. 7 8 9 10 11 12

Key words: Amyloid β peptide, Oligomers, Calcium, Bioluminescence imaging, Aequorin, Alzheimer's disease 13 14

1. Introduction 15

Alzheimer's disease (AD) is an aging-related neurodegenerative disorder associated to excess of amyloid β peptide ($\text{A}\beta$) production, an aberrant cleavage product of the amyloid precursor protein. AD has been also related to dis-homeostasis of intracellular Ca^{2+} , an elusive issue that remains controversial (1, 2). $\text{A}\beta$ species in AD brains are made of polypeptides of several lengths that may show different assemblies from monomers, dimmers, or other oligomers to the large fibrils forming macroscopic amyloid plaques, the hallmarks of AD (3). $\text{A}\beta$ species are toxic to neurons and contribute largely to neurodegeneration in AD. The mechanism of neurotoxicity is under intense scrutiny and might differ among the several species (4, 5). Recent data show that $\text{A}\beta_{1-42}$ oligomers, the most likely neurotoxin in AD, but not fibrils, induce dramatic increases in cytosolic and mitochondrial Ca^{2+} concentrations in primary 16 17 18 19 20 21 22 23 24 25 26 27 28 29

neurons that contribute to neurotoxicity (6). Here, we describe in detail the procedures for studying neurotoxic intracellular calcium signalling triggered by amyloids including neuron cell culture, preparation of different A β_{1-42} species including oligomers and fibrils as well as the methodology to investigate the effects of these species on cytosolic and mitochondrial Ca²⁺ in single neurons by fluorescence imaging of fura2-loaded cells and bioluminescence imaging of cells expressing aequorin targeted to mitochondria.

2. Materials

2.1. Cell Preparation, Culture, and Transfection

1. HEPES-buffered saline (HBS) contains 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, 10 mM sodium-HEPES, pH 7.4.
2. Ham's F12 nutrient mixture is made solving (powder) Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich Ref. 7777-10X1L) in 900 ml of double-distilled, sterile water. Then, 6 g HEPES and 336 mg NaHCO₃ are added to the mix. The solution is stirred and the pH adjusted to 7.42 using 4 N NaOH. Sterile water is added to a final volume of 1 l. Solution is then filtered in sterile conditions using Nalgene filters (Nalgene Labware, Roskilde, Denmark) and saturated with CO₂ in sterile conditions before use.
3. Hank's medium is prepared mixing 85 ml of HBSS medium (Gibco Ref. 041-04170) with 15 ml of 4% bovine serum albumin (BSA, prepared previously solving 4 g of BSA in 100 ml of HBSS).
4. Solutions for cell dissociation: Dispase II (neutral protease) from *Bacillus polymyxa*, grade II (Roche).
5. Culture medium is made of DMEM (Gibco Ref. 41966-029) containing 4,500 mg/l glucose and 2 mM L-glutamine, fetal bovine serum (FBS, 10%), horse serum (HS, 5%), penicillin 100 U/ml, and streptomycin 100 µg/ml. All these items are from Invitrogen, Barcelona, Spain.
6. Sato's medium (7) is made of DMEM (Gibco Ref. 41966-029) containing also 0.5% HS, 5 µg/ml insulin, 100 µg/ml BSA, 16 µg/ml putrescine, 30 nM Se²⁺, 60 ng/ml progesterone, 0.4 µg/ml tiroxine, 0.3 µg/ml tri-iodotironine, and 5 mg/ml holo-transferrin.
7. Poly-L-lysine-coated coverslips are prepared by placing 12-mm glass sterilized coverslips into a 0.01 mg/ml solution of poly-L-lysine (Sigma-Aldrich, Madrid, Spain). Treat for 15 min and then wash the coverslips twice with water and air-dry for 30–60 min under sterile conditions.

**2.2. Preparation of
Amyloid β Peptide
Species**

8. Multidish four wells for 12-mm glass coverslips are from Nunc (Ref. 176740), Rochester, NY, USA.	72
9. Amaxa Nucleofector and solution kit for neurons (Lonza Cologne, Germany).	74
Amyloid β peptide (1-42) is from Bachem AG (Bubendorf, Switzerland).	76
Solvents HCl and hexafluoroisopropanol (HFIP) are from Sigma-Aldrich.	78
Dimethyl sulfoxide (DMSO, Sigma-Aldrich).	80
DMEM F12 without phenol red (Gibco, Ref. 21041-025).	81
DMEM without phenol red (Lonza, Ref. 12-917F).	82
Speed Vac SPD111V (Thermo Electron Corporation, Marietta, OH, USA).	83

**2.3. Fluorescence
and Bioluminescence
Imaging**

1. HBS containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM sodium-HEPES, pH 7.4.	85
2. Fura2/AM (Invitrogen, F1201, 1 mg). Stock solutions are made in DMSO at a concentration of 2 mM and stored at $-20^\circ C$.	88
3. Digitonin (Sigma-Aldrich, D5628) dissolved at 100 μM in HBS containing 10 mM $CaCl_2$ instead 1 mM. Stock solution can be prepared at 10 mM in DMSO.	91
4. mGA plasmid. The mGA probe contain a mutated, low-affinity aequorin targeted to mitochondria and a GFP sequence to help select transfected neurons for bioluminescence imaging (8).	94
5. Coelenterazine <i>n</i> (Invitrogen, Ref. C6776). Stock solution is dissolved in methanol at 200 μM , aliquoted in 30 μl portions in Eppendorf tubes in ice and gassed briefly with N_2 before closing the tubes. Wrap with aluminum foil and store at $-80^\circ C$ for up to 6 months.	97
6. Zeiss Axiovert S100 TV (Carl Zeiss, Inc., Gottingen, Germany) inverted microscope equipped with a Zeiss Fluor 40x, 1.3 NA oil objective, a Xenon XBO75 fluorescence excitation lamp or a XCITE illumination system (EXFO, Ontario, Canada), a excitation filter wheel (Sutter Instrument Company, Novato, CA, USA) with band pass filters for fura2 excitation (340 and 380 nm) and a fura2 dichroic mirror.	102
7. ORCA ER fluorescence camera (Hamamatsu) mounted in the frontal or the lateral port of the microscope and handled by the Hamamatsu Aquacosmos software.	109
8. Hamamatsu VIM photon-counting ICCD camera (C2400-35) mounted in the bottom port of the microscope. Argus Image	112

Processor and M4314 image intensifier controller (Hamamatsu) are handled by the Aquacosmos software.

9. Cell perfusion system for living cells mounted in a PH-3 thermostated platform for open 12-mm glass coverslips using an 8-line gravity-driven perfusion system equipped with pinch valves (VC-8 valve controller) and solutions heated using an SH-27B inline heating system. All the above components are from Warner Instruments, Hamden, CT, USA.
10. Home-made, light-proof box covering the imaging set up. Size is about $100 \times 100 \times 100$ cm. They are also available from commercial sources (see Hamamatsu Photonics, Hamamatsu, Japan).

3. Methods

3.1. Neuron Cell Culture

1. Cerebellar granule cells are obtained from 5 to 7-day-old Wistar rat pups as reported previously (9). Animals are sacrificed by decapitation and the head is quickly washed in fresh HBS medium devoid of Ca^{2+} and glucose.
2. The skull is open to extract the brain that is washed quickly with Ham's F12 medium before dissecting the cerebellum.
3. Meninges are carefully removed and cerebellum is then washed with Hank's medium without Ca^{2+} or Mg^{2+} at 4°C .
4. Cerebellum is then cut in small pieces (about 2 mm) and incubated with Hank's medium without Ca^{2+} or Mg^{2+} but containing now dispase II (5 mg/ml) for 30 min at 37°C with discontinuous stirring every 5–10 min.
5. Tissue pieces are then dispersed using a silanized Pasteur pipette.
6. Dispersed tissue is allowed to rest to help debris removal. Supernatant-containing dispersed cells is placed into centrifuge tubes containing 7 ml of DMEM and cell suspension is then centrifuged at $200 \times g$ for 5 min.
7. The cell pellet is suspended in 1 ml of DMEM and then centrifuged again. Supernatant is removed and the new pellet is suspended again in 1 ml of DMEM containing now 10% horse serum.
8. At this point, cell density is estimated using a Neubauer counting chamber and medium is added to get a cell concentration of 1×10^6 cells/ml. At this point, 60 μl drops of cell suspension are plated on 12-mm glass coverslips previously coated with poli-L-lysine (0.01 mg/ml) and located inside the four wells multidish plaque. Cells are allowed to attach to the coverslips inside a cell incubator (37°C , 10% CO_2) for about 1 h.

9. Once cells are attached, 500 μ l of DMEM containing 156
4,500 mg/ml glucose, 10% FBS, 5% horse serum, and antibiotics 157
(penicillin, 100 U/ml and streptomycin, 100 μ g/ml) are 158
added to the four wells. 159
10. After 24 h, plating medium was exchanged for Sato's medium 160
(7) containing 5% HS to avoid excess proliferation of glia (9). 161
Cells are kept in the incubator for 3–5 days before imaging 162
experiments. 163

3.2. Preparation of A β Oligomers and Fibrils

1. Preparation of A β_{1-42} assemblies is carried out as described by 164
Klein (10). A β_{1-42} (Bachem AG) is initially dissolved to 1 mM 165
in hexafluoroisopropanol and aliquoted in sterile, non-siliconated 166
microcentrifuge tubes. The peptide was incubated 60 min at 167
room temperature and then in ice for 5–10 min. 168
2. The eppendorf containing the peptide solution is maintained 169
open overnight to allow evaporation of HFIP. 170
3. Hexafluoroisopropanol (HFIP) is then fully removed under 171
vacuum in a speed vac. (10 min), and the peptide film was 172
stored desiccated at -80°C . 173
4. For the aggregation protocol, the peptide was first solved in 174
dry DMSO at a concentration of 5 mM and treated differently 175
for preparation of oligomers and fibrils (Fig. 1). 176
5. For preparation of oligomers, DMEM without phenol red was 177
added to bring the peptide to a final concentration of 100 μ M 178
and incubated at 4°C for 24 h. The preparation was then 179
centrifuged at $14,000\times g$ for 10 min at 4°C to remove insoluble 180
aggregates (protofibrils and fibrils) and the supernatants 181
containing soluble A β_{1-42} oligomers were transferred to clean 182
tubes and stored at -20°C (see Note 1). 183
6. For preparation of fibrils, the peptide resuspended in DMSO at 184
5 mM concentration was diluted in 10 mM HCl to bring the 185
peptide to a final concentration of 100 μ M and incubated at 186
 37°C for 24 h (see Note 1). 187

3.3. Fluorescence Imaging of Cytosolic Ca $^{2+}$

1. Coverslips containing cultured cerebellar granule cells were 188
washed with HBS medium and incubated in the dark in the 189
same medium containing 4 μ M fura2/AM (11) for 60–90 min 190
at room temperature. 191
2. Coverslips are then placed on a Warner PH-3 thermostated 192
platform for open 12-mm glass coverslips (total volume about 193
500 μ l) on the stage of an inverted microscope (Zeiss Axiovert 194
S100 TV) and perfused continuously with heated (37°C) HBS 195
using a Warner 8-line gravity-driven perfusion system equipped 196
with pinch valves (Warner VC-8 valve controller) and a Warner 197
SH-27B inline heating system (see Note 2). 198

Fig. 1. Preparation of A β oligomers and fibrils. A β ₁₋₄₂ (Bachem AG, 1 mg) was dissolved at 1 mM in hexafluoroisopropanol (HFIP) and incubated first 60 min at RT and then 10 min in ice. Then, the peptide was incubated overnight with the eppendorf's cup open. HFIP was removed under vacuum in a speed vac., (10 min); the peptide film can be stored desiccated at -20°C at this point. The peptide film is solved in DMSO at 5 mM and treated differently for preparation of oligomers (*right*) and fibrils (*left*). For oligomers (*right*), DMEM F-12 without phenol red is added to bring the peptide to 100 μ M and incubated at 4°C for 24 h. Then, preparation is centrifuged (14,000 $\times$ g, 10 min, 4°C) and the supernatant-containing A β ₁₋₄₂ oligomers is transferred to clean non-siliconated, eppendorf tubes and stored at -20°C until use. For fibrils (*left*), the peptide (5 mM) in DMSO is solved in 10 mM HCl to bring the peptide to a final concentration of 100 μ M and incubated at 37°C for 24 h. Then, samples are stored at -20°C.

3. Cells are epi-illuminated alternately at 340 and 380 nm using band pass filters located on the excitation filter wheel. Light emitted above 520 nm at both excitation lights was filtered by the fura-2 dichroic mirror, collected every 5–10 s with a 40×, 1.4 NA, oil objective and recorded with the Hamamatsu Orca ER camera (Fig. 2).

Fig. 2. Equipment for imaging of cytosolic and mitochondrial calcium. The system set up for imaging is built on an inverted microscope (Zeiss Axiovert S100 TV). For fluorescence excitation, a X-cite excitation lamp (1) provides excitation light that passes through filters (340 and 380 nm) contained in a filter wheel (2) and reflected by a dichroic mirror (3) before illuminating cells located in the heated stage of the microscope (4). Fura2 fluorescence (>520 nm) is collected by a Zeiss Fluor 40x, 1.3 NA oil objective, passes through the dichroic mirror (3) but is reflected by a further mirror (5) before being captured by a frontal or lateral-port located, Hamamatsu Orca ER digital camera (6) handled by the camera controller and the aquacosmos software in a computer system (7). For bioluminescence imaging, photonic emissions coming from the living cells pass through the least possible optic devices (dichroic mirror is removed from the pathway) including only an analyzer lens and a tube lent. Light travels through the microscope's bottom port via adaptor without lent and reaches the Hamamatsu C2400-35 ICCD intensifier (8) controlled by the M431 image intensifier controller, before is captured by a Hamamatsu CCD video camera (9). Signal goes through an Argus image processor and a JVC RGB monitor and is finally processed by the same computer system (7). The entire microscope must be covered by a light-proof box (10) to avoid light interference. Cells are perfused using a gravity-fed system (Warner). Solutions are prewarmed at 37°C (11) and driven through silicon tubes (12) by a valve-controlled perfusion system (13). All tubes converge by a manifold system (14) into one silicon tube that goes through an on-line heating system (15) before getting to the stage. Medium is continuously removed by another silicon tube attached on the platform and aspirated by vacuum pump (16) to the waste bottle (17).

Fig. 3. $A\beta_{1-42}$ oligomers elicit increases in cytosolic Ca^{2+} in neurons. Cerebellar granule cells were cultured, loaded with fura2 and subjected to fura2 imaging. Pictures show pseudocolor images (Ratio 340/380) of granule cells before (left) and after stimulation with $A\beta_{1-42}$ oligomers. Warmer colors reflect increased cytosolic Ca^{2+} concentration. Recordings show cytosolic Ca^{2+} concentrations taken every 5 s in three individual cells. Perfused $A\beta_{1-42}$ oligomers increase cytosolic Ca^{2+} concentrations in all three neurons.

4. A background image is also captured at both excitation wavelengths with the shutter closed. During recordings, cells are perfused either with heated (37°C) control HBS or HBS containing test substances at a flow of 5 ml/min. This flow ensures that the medium bathing the cells is exchanged about ten times in less than 1 min.
5. At the end of the recording period, the complete sequences of images taken at 340 and 380 nm excitation light are stored in the computer for further analysis.
6. Using the Aquacosmos software, background images are subtracted and the resulting images are ratioed pixel by pixel to obtain a sequence of ratio images. Ratio images are coded in pseudocolor to better appreciate changes in Ca^{2+} concentrations. Perfusion of cerebellar granule cells with oligomers prepared from $A\beta_{1-42}$ induced rather dramatic increases in these ratios (Fig. 3), whereas no effect is induced by fibrils (data not shown).
7. For quantitative analysis of individual cells, regions of interest (ROIs) are drawn on individual cells and all ratio values corresponding to all pixels within each ROI are averaged for each image resulting in a recording of ratio values for individual ROIs (cells).
8. Recorded ratio values are converted into Ca^{2+} concentration values using the algorithm developed by Grynkiewicz et al. (11) (see Note 3).

**3.4. Bioluminescence
Imaging of
Mitochondrial Ca^{2+}**

1. Coverslips containing cerebellar granule cells are transfected with the mGA plasmid (see Note 4) using a Nucleofector II® device and the VPG-1003 transfection kit (Amaxa Biosystems, Cologne, Germany). Neurons are cultured for 24 h after transfection to allow efficient expression and targeting of the probe. 229 230 231 232 233 234
2. The coverslips containing the cells expressing the apoaequorins are transferred to a 4-well plate containing 0.20 ml of HBS. Then, 1 μ l of coelenterazine *n* is added and gently mixed. Finally, the plate is incubated for 1–2 h at room temperature in the dark. 235 236 237 238 239
3. Bioluminescence imaging was carried out as described previously (12, 13). Coverslips are placed on the stage of the bioluminescence microscope (Zeiss Axioverti S100 TV) into a perfusion chamber thermostated to 37°C and perfused continuously with prewarmed HBS at a rate of 5 ml/ml. The cells are examined for GFP fluorescence using the FITC filters and the microscopic field selected. A typical field may contain 4–8 transfected cells. A GFP fluorescence image (Fig. 4) is captured with the help of the Hamamatsu C2400-35 ICCD camera with the sensitivity set to a minimum (0) and located in the bottom port of the microscope (see Note 5). 240 241 242 243 244 245 246 247 248 249 250
4. After turning off the excitation lamp, a bright field image of the same cells is captured using the same camera. 251 252
5. Microscope light was turned off and the dark-box doors closed for complete darkness (see Note 6). Sensitivity of the intensifier is then set to maximum (12) and photonic emission images are captured with the Hamamatsu VIM photon-counting camera handled with an Argus-20 image processor and integrated every 10 s periods (Fig. 4). Total counts per region of interest may range between 2×10^3 and 2×10^5 for different cells. Background photonic emissions in regions of interest of similar size in nonexpressing cells are typically about 1 ± 1 count per second per typical cell area (about 2,000 pixels). 253 254 255 256 257 258 259 260 261 262
6. Cells are perfused at selected periods with A β species at an estimated concentration of 1 μ M by using the perfusion system. Alternatively, A β species can be provided as drops using an automatic pipette. 263 264 265 266
7. Before ending the experiment, cells are perfused with HBS containing 0.1 mM digitonin and 10 mM $CaCl_2$ to permeate the cells and release all the remaining aequorin counts. These photonic emissions must be added up to estimate the total photonic emissions, a value required for calibration in Ca^{2+} concentrations. Emission of all residual counts may take 2–5 min from the time of lysis. The experiment is finished once aequorin photonic emissions cease. 267 268 269 270 271 272 273 274

Fig. 4. $A\beta_{1-42}$ oligomers induce mitochondrial Ca^{2+} overload in neurons. Cerebellar granule cells were transfected with the low-affinity, mitochondria-targeted aequorin fused to GFP, incubated with 1 μM coelenterazine *n* and subjected to bioluminescence imaging of $[Ca^{2+}]_{mit}$. Pictures show the fluorescence (top, GFP fluorescence) and accumulated photonic emissions (bottom, Aequorin Bioluminescence) images of representative microscopic fields. Luminescence intensity is coded in pseudocolor. Top recordings show the release of photonic emissions (expressed as % of remaining counts) during recording in four individual cells. Perfusion with $A\beta_{1-42}$ oligomers (but no fibrils) released photonic emissions (thus, decreasing the % of remaining counts). Those values were used to calculate mitochondrial Ca^{2+} concentrations (bottom recordings). $A\beta_{1-42}$ oligomers promoted increases in mitochondrial Ca^{2+} concentrations that reached even the mM level as previously reported (6).

8. Bioluminescence images are finally stored in the computer with the bright field and fluorescence images captured before photon-counting imaging.
9. Regions of interest (ROIs) are selected with the help of the Aquacosmos software by drawing circles around infected cells according to the fluorescence and bright field images captured at the beginning of the experiment (see Note 7). The same ROIs are pasted on every image of the sequence. Total photonic emissions in every ROI are computed with the Aquacosmos software to obtain the luminescence emission value (L) for each cell at each point in time. A few ROIs are drawn in regions devoid of cells to compute background luminescence.
10. All the photonic emissions in the bioluminescence images, including the obtained after digitonin permeabilization, are added up using the Aquacosmos software to obtain a bioluminescence image containing all the photonic emissions. The size of the ROI is adjusted to the area in which photons are emitted from each individual cell, which is usually somewhat larger than the size of the cell. Cells with overlapping of photonic emissions are excluded from the analysis.
11. The luminescence values for every ROI at each time value (L) are computed and exported to a worksheet. Background luminescence is subtracted from each L value. For every ROI, the total luminescence, $Total(L)$ was computed by adding up the values of all the images. Then, the following values were computed for every time point (t):
 - L : Luminescence emission at time t
 - $Sum(L) = \sum L$ values from $t=0$ to t
 - $LTOTAL$: Total luminescence remaining at time $t = Total(L) - Sum(L)$
 - % Remaining luminescence: $100 \times LTOTAL / Total(L)$
 - $L / LTOTAL$
 - $[Ca^{2+}]$ using the following algorithm:

$$[Ca^{2+}] \text{ (in M units)} = \frac{R + (R \times K_{TR}) + 1}{K_R - (R \times K_R)},$$

$$\text{where } R = \frac{L}{L_{TOTAL} \times \lambda^{(1/n)}},$$

where L is the luminescence emitted at the time of measurement and L_{TOTAL} is the addition of the counts present in the tissue at that time, estimated by integrating all the counts from the time of measurement until the release of all the residual luminescence by lysis at the end of the experiment. Calculations were made using the constant values published elsewhere (14).

4. Notes

317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338

1. Characterization of amyloid β preparations is beyond the scope of this chapter. However, the characterization is required to confirm the species that are present in each preparation, their purity and actual concentrations. This characterization is not simple and may require a combination of several approaches including electron microscopy, amino acid analysis, PAGE-SDS, and silver staining (6).
2. Perfusion is very helpful during imaging not only to add and remove easily and quickly test solutions to the cell chamber but also to keep physiological conditions (37°C, or even CO₂ if required) thanks to the in-line heating system. Perfusion of expensive test solutions such as A β species may be, however, cost limiting. In those cases where perfusion is not available or convenient, A β can be provided by carefully adding a 250 μ l drop of 2 $\times$, A β -containing solution, to a half-filled chamber (about 250 μ l) and making a quick mix of solutions. In this case, it is best to keep the heating system off and make the experiment at room temperature to avoid changes in the composition of saline due to evaporation.
3. Binding of Ca²⁺ to fura2 changes fura2 fluorescence according to the law of mass action (11):

$$[\text{Ca}^{2+}] = K_d \frac{F_{\max} - F}{F - F_{\min}},$$

339
340
341
342
343
344

where K_d is the dissociation constant of the fura2–Ca²⁺ complex (224 nM at 37°C), F is the fluorescence emission for each [Ca²⁺], $F_{\max}$ is the fluorescence emission when fura2 is saturated with Ca²⁺, and $F_{\min}$ is fluorescence emission when fura2 is free of Ca²⁺. If we apply the above algorithm to both wavelengths and do the ratio, then we obtain the following algorithm:

345

$$[\text{Ca}^{2+}] = K_d \beta \frac{R_{\max} - R}{R - R_{\min}},$$

346
347
348
349
350
351
352
353

where R is the ratio of fluorescence recordings obtained after exciting at 340 and 380 nm for a given [Ca²⁺], $R_{\max}$ is the same ratio when fura2 is saturated with Ca²⁺, and $R_{\min}$ is the same ratio when fura2 is free of Ca²⁺. Finally, β is the ratio of $F_{\max}/F_{\min}$ at 380 nm. This algorithm allows estimation of [Ca²⁺] knowing the R values at any point in time. $R_{\max}$, $R_{\min}$, and β values can be determined experimentally using fura2 solutions in the presence of saturating concentrations of Ca²⁺ (HBS containing

- 1 mM Ca^{2+}) and in the absence of Ca^{2+} free medium (HBS without added Ca^{2+} and containing 5 mM EGTA).
4. The use of the fusion protein GFP–aequorin in the mGA plasmid has many advantages over the native aequorin. It allows direct visualization of the expressed protein under the fluorescence microscope which is very convenient particularly when transfection efficiency is low. In addition, the protein is more stable, and it gives a higher quantum yield (8).
5. The same set up for fluorescence imaging can be used for bioluminescence imaging. We have the fluorescence camera placed in the lateral port and the photon-counting camera in the bottom port. This configuration avoids losing of aequorin photonic emissions when the system is used in the bioluminescence imaging mode. Before photon counting, a GFP fluorescence image of GFP and aequorin-transfected cells is captured with the bottom-port located, photon-counting camera and the sensitivity gain set to a minimum. Then, the dichroic-containing box located in the light pathway is removed, the excitation light turned off, the dark-box doors are closed and the sensitivity gain is set to a maximum before starting bioluminescence imaging.
6. Photon-counting cameras are extremely sensitive to any contaminating light. Accordingly, any light source inside the dark box must be turned off during photon counting. Putative light sources such as leads of different operators, even if they are outside the dark box, must be turned off. Some motorized microscopes have internal leads that may produce a light leak to the camera.
7. Calibration of the recorded signals is dependent of the total amount of photonic emissions, including those released after cell permeabilization. If cell density is excessive or the amount of light emitted by some individual cells is too high, overlap between photonic emissions from different cells may happen and this could influence the calculations of Ca^{2+} concentrations. This can be avoided by optimizing expression efficiency and/or by plating the cells at a proper density.

Acknowledgments

Financial support from Spain's Ministerio de Educación y Ciencia (BFU2009-08967), Instituto de Salud Carlos III (PI07/0766), and Junta de Castilla y León (CSI12A08 and VA270A11-2) are gratefully acknowledged.

References

1. Berridge, M. J. (2010) Calcium hypothesis of Alzheimer's disease. *Pflugers Arch.* **459**, 441–449.
2. Foskett, K., Bacsai, B., Kuchibhotla, K., Fagan, T., Bezprozvanny, I., Moyer, J., Stutzmann, B., Green, K., Villalobos, C., Michno, K., Goussakov, I., Atwood, C., Rybalchenko, V., Chakroborty, S. (2009) Alzheimer research forum live discussion: calcium in AD pathogenesis. *J. Alzheimers Dis.* **16**, 909–917.
3. Haass, C., Selkoe, D. J. (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat. Rev. Mol. Cell Biol.* **8**, 101–112.
4. Dahlgren, K. N., Manelli, A. M., Stine, W. B. Jr, Baker, L. K., Krafft, G. A., LaDu, M. J. (2002) Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *J. Biol. Chem.* **277**: 32046–32053.
5. Deshpande, A., Mina, E., Glabe, C., Busciglio, J. (2006) Different conformations of amyloid beta induce neurotoxicity by distinct mechanisms in human cortical neurons. *J. Neurosci.* **26**, 6011–6018.
6. Sanz-Blasco, S., Valero, R. A., Rodríguez-Crespo, I., Villalobos, C., Núñez, L. (2008) Mitochondrial Ca^{2+} overload underlies A β oligomers neurotoxicity providing an unexpected mechanism of neuroprotection by NSAIDs. *PLoS ONE* **3**(7): e2718
7. Botteststein, J.E., Sato, G. H. (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA* **76**, 514–517.
8. Rogers, K. L., Stinnakre, J., Agulhon, C., Jublot, D., Shorte, S. L., Kremer, E. J., Brûlet, P. (2005) Visualization of local Ca^{2+} dynamics with genetically encoded bioluminescent reporters. *Eur. J. Neurosci.* **21**, 597–610.
9. Núñez, L., Sánchez, A., Fonteriz, R. I., García-Sancho, J. (1996) Mechanisms for synchronous calcium oscillations in cultured rat cerebellar neurons. *Eur. J. Neurosci.* **8**, 192–201.
10. Klein, W. L. (2002) Abeta toxicity in Alzheimer's disease: globular oligomers (ADDLs) as new vaccine and drug targets. *Neurochem. Int.* **41**, 345–352.
11. Grynkiewicz, G., Poenie, M., Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440–3450.
12. Villalobos, C., Núñez, L., Chamero, P., Alonso, M. T., García-Sancho, J. (2001) Mitochondrial $[\text{Ca}^{2+}]$ oscillations driven by local high $[\text{Ca}^{2+}]$ domains generated by spontaneous electric activity. *J. Biol. Chem.* **276**, 40293–40297.
13. Núñez, L., Senovilla, L., Sanz-Blasco, S. I., Chamero, P., Alonso, M. T., Villalobos, C., García-Sancho, J. (2007) Bioluminescence imaging of mitochondrial calcium in sympathetic neurons from adult rat dorsal root ganglion. *J. Physiol.* **580**, 385–395.
14. Villalobos, C., Alonso, M. T., García-Sancho, J. (2009) Bioluminescence imaging of calcium oscillations inside intracellular organelles. *Methods Mol. Biol.* **574**, 203–214.