Research

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Expression of apo-aequorin during embryonic development; how much is needed for calcium imaging?

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Summary Aequorin is a bioluminescent calcium indicator consisting of a 21 kDa protein (apo-aequorin) that is covalently linked to a lipophilic cofactor (coelenterazine). The aequorin gene can be expressed in a variety of cell lines and tissues, allowing non-invasive calcium imaging of specific cell types. In the present paper, we describe the possibilities and limitations of calcium imaging with genetically introduced apo-aequorin during embryonic development. By injecting aequorin into sea urchin, *Drosophila* and zebrafish eggs, we found that higher aequorin concentrations are needed in smaller eggs. Our results suggest that for measuring resting levels of free cytosolic calcium, one needs aequorin concentrations of at least 40 μM in sea urchin eggs, 2 μM in *Drosophila* eggs, and only 0.11 μM in zebrafish eggs. A simple assay was used to determine the absolute concentrations of expressed apo-aequorin and the percentage of aequorin formation in vivo. The use of this assay is illustrated by expression of the aequorin gene in *Drosophila* oocytes. These oocytes form up to 1 μM apo-aequorin. In our hands, only 0.3% of this apo-aequorin combined with coelenterazine entering from the medium to form aequorin, which was not enough for calcium imaging of the oocytes, but did allow in vivo imaging of the ovaries. From these studies, we conclude that coelenterazine entry into the cell is the rate limiting step in aequorin formation. Based on the rate of coelenterazine uptake in *Drosophila*, we estimate that complete conversion of 1 μM apo-aequorin would take 50 days in zebrafish eggs, 19 days in *Drosophila* eggs, 7 days in sea urchin eggs or 18 h in a 10 μm tissue culture cell. Our results suggest that work based on genetically introduced apo-aequorin will be most successful when large amounts of small cells can be incubated in coelenterazine. During embryonic development this would involve introducing coelenterazine into the circulatory system of late stage embryos. Calcium imaging in early stage embryos may be best done by injecting aequorin, which circumvents the slow process of coelenterazine entry.

INTRODUCTION

Aequorin is a bioluminescent calcium indicator originally isolated from the jelly fish *Aequorea aequorea* [1]. It consists of a 21 kDa protein (apo-aequorin) which is covalently linked to a cofactor (coelenterazine) which acts as the luminophore [2–4]. The aequorin gene has been introduced into bacteria [5], yeast [6], plants [7], slime molds [8,9], fruit flies [10] and mammalian cell lines [11,12], allowing non-invasive calcium imaging of specific cell types and cell organelles [13,14]. Expressed apo-aequorin needs to be reconstituted into aequorin by adding coelenterazine to the culture medium. The lipophilic coelenterazine diffuses through the plasma membranes and becomes covalently linked to apo-aequorin. Only then is the aequorin molecule ready for calcium detection. When calcium ions bind to the aequorin molecule, coelenterazine is oxidized and a quantum of blue light (466 nm) is emitted [4,15]. The usual ultra low levels of light are easily recorded with widely available photomultiplier tubes. However, spatial information can be best obtained with a special system based on an imaging photon detector [16].

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Aequorin is a powerful indicator to study calcium patterns in embryonic development. By directly injecting aequorin into uncleaved eggs, two classes of calcium waves were discovered: fast waves which activate eggs during fertilization and slow ones which control cell division [17,18]. Moreover, the non-invasive nature of imaging with aequorin allows calcium measurements during prolonged periods of time. In zebrafish development, it was possible to image calcium patterns continuously for more than 24 h and thus during formation of the somites, the brain, the eyes and the heart [19]. The expression of apo-aequorin could provide new insights in calcium signaling during embryonic development. For example, apo-aequorin could be expressed in otherwise inaccessible cells such as early stage oocytes which reside in the ovaries. Moreover, apo-aequorin could be expressed locally, for instance in specific germ layers or in specific regions of the developing brain. Such local expression would avoid confusion by signals from surrounding tissues and allows a clear distinction between calcium increases in inducing and receptive cells. In the present paper, we describe how much aequorin is needed for calcium imaging in different size embryos. A simple assay was used to estimate apo-aequorin concentrations and aequorin formation rates in vivo. The assay was illustrated by stable apo-aequorin expression in *Drosophila* oocytes. These oocytes express up to 1 μM apo-aequorin. Only 0.3% of this apo-aequorin combined with externally applied coelenterazine to form aequorin, which was not enough for calcium imaging of the oocytes but did allow in vivo imaging of the ovaries.

MATERIALS AND METHODS

Handling of embryos

Eggs and sperm of the sea urchin *Lytechinus variegatus* were obtained by electrical stimulation of the urchins. The eggs were dejellied by a 3 min treatment in pH 5 sea water and were fertilized in sea water, pH 8. Fertilized *Drosophila* eggs were collected from apple juice agar plates. The Oregon stock was used for aequorin injection studies, the w^{1118} stock was used for aequorin expression studies. Before injecting aequorin, the eggs were dechorionated with tape, dried for 5 min, and covered with halocarbon oil [20]. Fertilized zebrafish eggs were collected from the aquarium 30 min after 'dawn' [21]. The embryos were left inside their chorion and were cultured at 28°C in spring water containing 1 mg/l methylene blue.

Microinjection of aequorin

Various embryos were injected with 0.25 mM *h*-aequorin dissolved in a buffer containing 100 mM KCl, 0.05 mM EDTA and 5 mM MOPS at pH 7. The *h*-aequorin was kindly provided by Dr Shimomura [2], who reconstitutes recombinant apo-aequorin [22] with different types of coelenterazine. For example, reconstitution with natural coelenterazine gives aequorin, whereas the semisynthetic *h*-coelenterazine is used to produce *h*-aequorin. Thus, the *h*-aequorin belongs in the class of semisynthetic recombinant aequorins [2]. Typically, 1% of the egg volume was injected, resulting in a final aequorin concentration of 2.5 μM in the egg. The amount of injected aequorin was determined by first injecting droplets of aequorin under oil. This allows one to measure the diameter of the injected droplet. The injection pressure was adjusted until the desired injection volume was achieved. Cytosolic aequorin concentrations are approximately 5 μM, since aequorin is not present in the cell organelles. In the present paper, aequorin concentrations refer to the concentration in the egg rather than the concentration in the cytosol. For the sea urchin egg, we used a low pressure injection system [16]. For *Drosophila* and zebrafish eggs, we used a PLI-100 high pressure injection system from Medical Systems Co. (Greenvale, NY, USA) [16]

Calcium imaging

The dim aequorin light was efficiently gathered with a Zeiss Axiovert 100-TV microscope, using either a 20× objective (NA = 0.75) for the zebrafish eggs, or a 40× objective (NA = 1.3) for the urchin and *Drosophila* eggs. To minimize instrumental noise and allow recording over many hours without overloading memory, we use the imaging photon detector (IPD) made by Photek Inc., East Sussex, UK. This instrument stores images as a list of photon events, each photon having two space coordinates (x,y) and one time coordinate. The two space coordinates identify an individual pixel in the imaging field and with the 20× objective, each pixel corresponds to an area of 10 × 10 μm. The digital storage of photons allows flexible reviewing of the calcium patterns. Thus images of light emitted over any desired period can be recreated in pointillist style [16].

Transformation of *Drosophila*

The apo-aequorin expressing flies were kindly provided by Dr Douglas Robinson and Dr Lynn Cooley at Yale University. In creating the flies, Dr Robinson subcloned aequorin cDNA (AEQ1, provided by Dr Douglas Prasher [23]) into the *Drosophila* germline expression vector pCOG. This vector contains the ovarian tumor (otu) promoter that drives gene expression throughout oogenesis [24]. The pCOG–aequorin construct was microinjected into w^{1118} embryos and transformants were recovered based on complementation with the *white*

mini-gene in pCOG. Eleven lines were stably transformed, each with a different insertion site in the genome.

The apo-aequorin assay

To fully convert genetically made apo-aequorin into aequorin, one freshly laid *Drosophila* embryo was homogenized in 20 µl of reconstitution buffer, containing 12 µM coelenterazine, 2% mercaptoethanol, 1 mM EGTA, 300 mM KCl and 10 mM Tris, pH 8. Coelenterazine was obtained from Molecular Probes in quantities of 0.25 mg and dissolved in 250 µl methanol giving a 2.4 mM stock. After a 3 h incubation at 4°C, the homogenate was transferred to a transparent vial and placed on a Hamamatsu (R464) photomultiplier tube (Bridgewater, NJ, USA). To burn out the reconstituted aequorin, we added 80 µl of a calcium buffer, containing 2 mM $CaCl_2$, 3 mM dibromo-BAPTA, 300 mM KCl, and 10 mM Tris, pH 8. This buffer sets the free calcium concentration in the homogenate at 5 µM. The recorded peak of luminescence was integrated, giving the total number of recorded photons. The total number of photons is a measure of how much apo-aequorin was present in the homogenate. The apo-aequorin assay was calibrated with known amounts of apo-aequorin.

In vivo formation of aequorin

For this purpose, we incubated live dechorionated *Drosophila* eggs for 4 h in 50 µM coelenterazine in IMADS buffer [25]. After the 4 h incubation, the eggs were rinsed in IMADS and were observed with the aid of a photomultiplier tube and an imaging photon detector. The same procedure was used for oocytes and ovaries which were dissected from an adult fly. At the end of each experiment, the eggs were permeabilized with Triton to burn out all the reconstituted aequorin. From these burnouts we can calculate the efficiency of reconstitution in vivo.

RESULTS AND DISCUSSION

How much aequorin is needed for calcium imaging?

Calcium imaging with aequorin is generally a struggle for light. Thus, it is desirable to have as much aequorin in the egg as possible. We estimated how much aequorin needs to be minimally expressed to still allow calcium imaging. To this end, we injected known amounts of the ultra sensitive *h*-aequorin into the eggs of sea urchins, fruit flies and zebrafish. The eggs were imaged with the IPD and the resulting luminescence was compared with instrumental background noise, which is approximately 0.5 photons/s over the embryonic field (Fig. 1). In

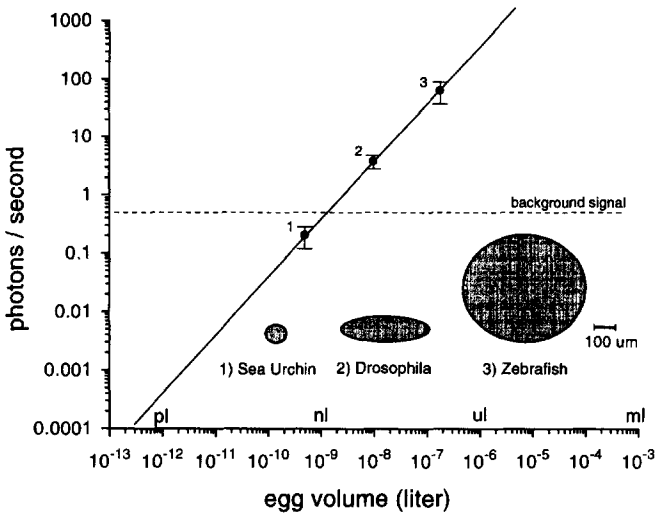
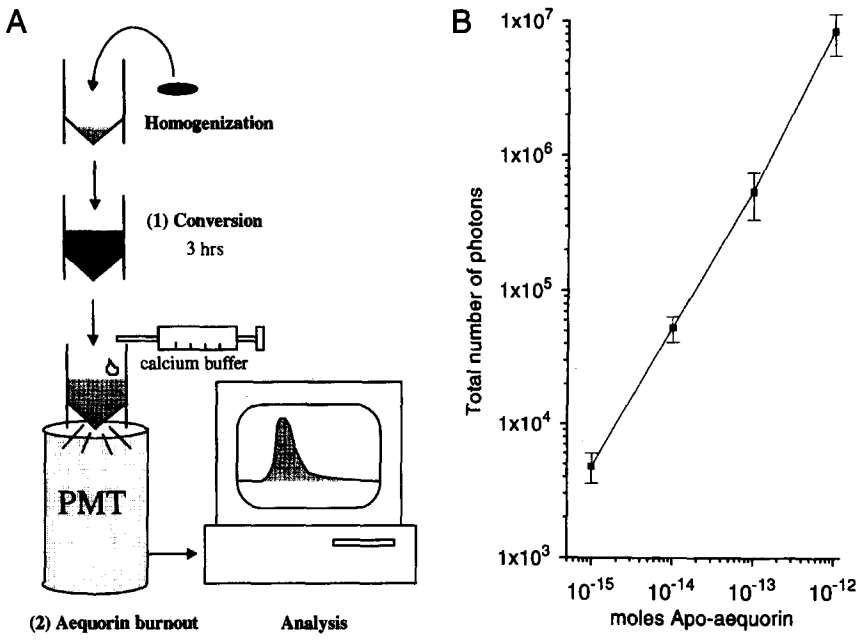


Fig. 1 Luminescence measured with the imaging photon detector of different size embryos at a resting level of about 100 nM free calcium. The embryos were injected with *h*-aequorin to yield a final concentration of 2.5 µM *h*-aequorin in the egg. The embryos emit luminescence at about 0.4 photons/s for each nl of egg volume. Background noise is 0.5 photons/s over the image field of one embryo (70 × 70 pixels). Thus, the large zebrafish egg (700 µm in diameter) is more than 300 times brighter than the sea urchin egg (100 µm in diameter). The background luminescence of the IPD is indicated by a dotted line (total luminescence in experiments is embryonic luminescence plus background).

analysing the different levels of luminescence, we looked at 'resting' embryos, i.e. ones that are in early stages of development, but do not show gross morphological changes such as secretion, cleavage, or contractions. Typically, the free calcium concentrations of such resting cells is on the order of 100 nM. We found that the resting level of luminescence is proportional to the volume of the egg (Fig. 1). Other factors such as the use of different objectives and differences in egg transparency do influence the level of luminescence, but to a lesser extent. For example, the 40× objective used for the *Drosophila* egg yields 3 times more light than the 20× objective used for zebrafish eggs. This effect is counteracted by the opaqueness of the *Drosophila* egg, which reduces the level of emitted light. To estimate how much light is lost by the opaque contents of the *Drosophila* egg, we compared the luminescence of BAPTA-injected embryos with the luminescence of BAPTA droplets in the same shape and size of a *Drosophila* embryo (data not shown). The droplets and embryos were both clamped at 100 nM free calcium and had the same amount of aequorin present. We found that the transparent droplets were 2.5 times brighter than the *Drosophila* eggs, suggesting that 60% of the light is lost by the opaque contents of the *Drosophila* egg. Thus, several factors will effect the level of luminescence when comparing different embryos. The 2–3-fold difference caused by



1. Apo-aequorin + Coelenterazine + O_2 ⟶ Aequorin

2. Aequorin $\xrightarrow{2\,Ca^{++}}$ Apo-aequorin + Coelenteramide + CO_2 + light (466 nm)

Fig. 2 A simple assay to quantify available concentrations of apo-aequorin. (**A**) An apo-aequorin expressing egg is homogenized and the homogenate is incubated in 'reconstitution medium' containing coelenterazine. After 3 h, the aequorin that forms is burned up by adding a calcium buffer; emitted light is measured with a photomultiplier tube. The area under the peak is used to calculate how much aequorin was present in the homogenate. The assay takes only a few minutes per sample, allowing mass screening of transgenic lines. (**B**) Calibration curve correlating integrated luminescence with known amounts of apo-aequorin. When calculating apo-aequorin concentrations in an embryo divide moles of aequorin by egg volume (e.g. 10 nl in *Drosophila*).

different objectives/differences in transparency is negligible when considering the effect of volume, i.e. an egg that is 10 times larger in diameter gives 1000-fold more light.

Since the different embryos all contained about 2.5 μM *h*-aequorin, we conclude that it is not the concentration, but the absolute amount of aequorin that determines light emission. The quality of the image is directly related to the amount of emitted light, because the emitted light is focused on a similar area of the detector, regardless of the cell size. In general, we focus an image of the embryo on a quarter of the detector field by using different magnifications for different size eggs. Thus, different size eggs have to overcome a similar amount of instrumental noise (0.5 photons/s over a quarter of the detector field). To estimate the minimum amount of aequorin necessary for calcium imaging, we refer to the sea urchin embryo, in which imaging of resting calcium levels is just possible. With an aequorin concentration of 2.5 μM and an egg volume of 0.5 nl, the total amount of *h*-aequorin is 1.25×10^{-15} moles or about 1 femtomole. Since it is only the total amount of *h*-aequorin that matters for imaging, this minimum of 1 femtomole is valid for all eggs and probably for other cell types as well. The *h*-aequorin used is an ultra sensitive one giving 16 times more light than the regular aequorin at resting levels of free calcium [2]. Thus 16 times more regular aequorin (2×10^{-14} moles or 20 femtomoles) would be required for imaging resting levels of calcium. These 20 femtomoles correspond to aequorin concentrations of 40 μM in sea urchin eggs, 2 μM in *Drosophila* eggs or 0.11 μM in zebrafish eggs. Based on our measurements in eggs, we extrapolate that a typical tissue culture cell with a diameter of 10 μm and a volume of 0.5 pl would need 40 000 μM (40 mM!) aequorin for calcium imaging. Our estimates are based on resting levels of calcium. Less aequorin would be needed to image high calcium levels.

In conclusion, when imaging resting levels of calcium one needs at least 20 femtomoles of regular aequorin. This amount is valid for different eggs and possibly for

other cell types as well. The minimum amount of aequorin is based on our imaging photon detector which is probably the most sensitive one currently available. The sensitivity of charge-coupled device (CCD) cameras containing microchannel plate intensifiers may be comparable. CCD cameras without microchannel plate intensifiers will be considerably less sensitive and thus require several magnitudes more aequorin in the specimen.

A simple assay for measuring apo-aequorin concentrations

To measure the amount of apo-aequorin formed in the eggs of transformed *Drosophila* lines, we homogenized one egg, incubated the homogenate in coelenterazine, and burned out the reconstituted aequorin on the photomultiplier tube [3,13,14]. The assay was calibrated with known amounts of apo-aequorin (Fig. 2). This apo-aequorin assay is fast, quantitative and can be used in mass screening of transgenic lines.

Apo-aequorin concentrations in transgenic *Drosophila*

Transformation with the aequorin gene resulted in 11 *Drosophila* lines that had the aequorin gene stably integrated in the genome. The aequorin gene was driven by the ovarian tumor promoter, so that apo-aequorin would be present in the germline cells as well as the freshly laid eggs. Only 3 out of 11 lines showed detectable levels of apo-aequorin (Fig. 3). Using the calibration curve of Figure 2, we calculated that our best line (#17-1) had about 1 μM apo-aequorin available in the egg. This apo-aequorin concentration is 10 times higher than the apo-aequorin concentration found in mammalian cell lines [14], but was still 2.5 times lower than the *h*-aequorin concentration obtained with injection. Even if this were fully converted into aequorid,, it would not be enough to measure resting levels of calcium in live *Drosophila* eggs, but 1 μM aequorin should be more than enough for imaging when calcium is elevated. Thus the next problem was to convert the genetically formed apo-aequorin into aequorin by applying coelenterazine.

The use of different coelenterazines for in vivo aequorin formation

When forming aequorin from apo-aequorin, different coelenterazines can be used. For example, *h*-coelenterazine gives *h*-aequorin, *e*-coelenterazine gives *e*-aequorin and the natural coelenterazine gives aequorin (also called recombinant or R-aequorin when recombinant apo-aequorin is used). In choosing the optimal type of coelenterazine three features need to be considered.

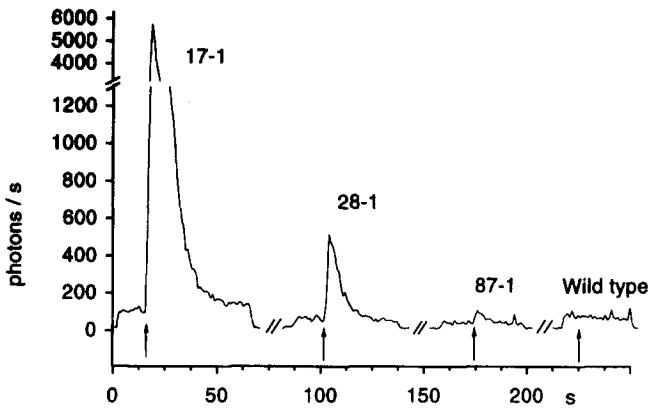


Fig. 3 Quantification of apo-aequorin expression in *Drosophila* eggs. Representative recordings show homogenized *Drosophila* eggs in the apo-aequorin assay. Arrows indicate the time points at which the calcium buffer was added to the homogenate. (**A**) *Drosophila* line #17-1 contains 0.85 (± 0.13) μM apo-aequorin in the egg. (**B**) *Drosophila* line #28-1 contains 0.14 (± 0.04) μM apo-aequorin in the egg. (**C**) *Drosophila* line #87-1 contains 0.04 (± 0.01) μM apo-aequorin in the egg. (**D**) Wild type eggs show no increase in luminescence when adding the calcium buffer.

Sensitivity of the aequorin formed

The sensitivities of different aequorins have been described by Shimomura et al [2]. For example, *h*-aequorin is 16 times more sensitive than recombinant aequorin and *e*-aequorin is 6 times more sensitive than recombinant aequorin. Even though *h*-aequorin is 16 times brighter than recombinant aequorin, it may not be a good choice for in vivo use because of slow formation rates and a short half life.

Aequorin formation rate

The in vitro 'reconstitution' rates for different aequorins have been described as well [3]. The 50% reconstitution times are 22 min for recombinant aequorin, 210 min for *h*-aequorin and 8 min for *e*-aequorin at 5°C. These rates were twice as fast at 24°C, thus about 11 min for recombinant aequorin, 105 min for *h*-aequorin, and 4 min for *e*-aequorin.

Aequorin half-life in vivo

For the half-life of aequorin in vivo, we refer to our own observations in zebrafish (data not shown). At the end of a zebrafish experiment, we elevated cytosolic calcium concentrations which burns up the remaining aequorin. From these burnouts we learned that 71% (± 9%) of the injected aequorin was still available for calcium imaging after 24 h of embryonic development. This corresponds to an in vivo half-life of 48 h for recombinant aequorin. More sensitive aequorins should have proportionally shorter half-lives, e.g. 8 h for *e*-aequorin and only 3 h for *h*-aequorin.

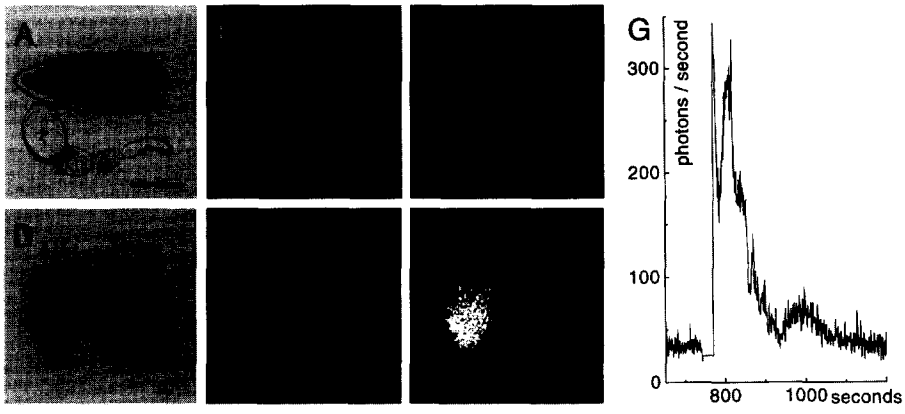


Fig. 4 Imaging luminescence of oocytes and ovaries from apo-aequorin expressing *Drosophila* lines. (**A**) Bright field picture showing a string of oocytes from *Drosophila* line #17-1. (**B**) Same oocytes, alive, under the imaging photon detector. No luminescence above the background levels could be detected. (**C**) The same oocytes show very little luminescence (a total of 1200 photons) when aequorin is burned out by addition of Triton. (**D**) Bright field image showing an ovary of wild type (WT) and an ovary of a *Drosophila* line #17-1, which expresses apo-aequorin. (**E**) Photon image of the same two ovaries in vivo. The apo-aequorin expressing ovary emits low levels of light. (**F**) During the burnout, bright signals are observed from the aequorin expressing ovaries. (**G**) These burnouts average only 28 000 (± 4000) photons on the photomultiplier tube, showing that a fraction of the apo-aequorin is reconstituted with coelenterazine. Scale bars are 100 μm, exposure times of 30 min. The gray scale in the photon images ranges from black (0 milliphotons/pixel.s) to white (10 milliphotons/pixel.s).

To avoid decay during the incubation, we used natural coelenterazine. The recombinant aequorin that is formed in this way, has a 50% conversion time of only 11 min and a half-life of 48 h. Thus, recombinant aequorin is formed rapidly and remains in the cells for prolonged periods of time. A second option would have been to use *e*-coelenterazine; *e*-aequorin forms even faster (50% conversion in 4 min), is 6 times more sensitive than recombinant aequorin and has an intermediate half-life of 8 h. An additional advantage of *e*-aequorin is the possibility of ratio imaging at 405/472 nm [3].

Aequorin formation in vivo

Apo-aequorin expressing *Drosophila* eggs and oocytes were soaked for 4 h in 50 μM natural coelenterazine, rinsed in medium without coelenterazine and imaged on the IPD. The eggs and oocytes showed no light in vivo, and very little light when calcium was elevated by adding Triton (Fig. 4A–C). Based on the formation rates of aequorin in vitro, more than 99% of the apo-aequorin (1 μM) should have been converted into aequorin, which should have given extremely bright signals when adding Triton. The lack of light during the burnout suggests that diffusion of coelenterazine through the plasma membranes is a limiting factor.

To estimate the rate of entry, we incubated *Drosophila* ovaries for 4 h in 50 μM coelenterazine, rinsed the ovaries in medium without coelenterazine, and imaged the ovaries on the IPD. One ovary is about 700 μm in diameter (volume = 180 nl) and is thus expected to have 18 times more apo-aequorin (180 femtomoles) and yield 18 times more light than a *Drosophila* egg. The ovaries showed low but detectable levels of light when imaged in vivo (Fig. 4E). As shown in Figure 4E, the coelenterazine gives some background signal in the wild type ovaries. This coelenterazine background is 3.5 times above the instrumental noise. The luminescence of the apo-aequorin expressing ovaries (#17-1) is 1.6 times higher than the controls (wild type oocytes incubated in coelenterazine). High levels of luminescence were observed, when calcium levels were raised with Triton into the millimolar range (Fig. 4F). From burnouts on the photomultiplier tube (Fig. 4G), we were able to determine the total amount of aequorin formed in the ovary. The burnouts averaged 28 000 (± 4000) photons which corresponds to 0.6 femtomoles of aequorin (see Fig. 2). The fact that we were able to image live ovaries with such low amounts of aequorin suggests that calcium was elevated in the dissected ovaries. Since the ovaries have about 180 femtomoles of apo-aequorin and only 0.6 femtomoles of reconstituted aequorin, the conversion process must have been very inefficient. In fact, only 0.33% of the available apo-aequorin is converted to aequorin in vivo!

Cell size hypothesis

The slow uptake of coelenterazine is in sharp contrast with the fast conversion rates in vitro and suggests that coelenterazine uptake through the plasma membrane is the limiting factor in the formation of aequorin. When

Table Size, volume and surface of various cells and 100% conversion times based on aequorin formation rates in *Drosophila*

	Cultured cell[a]	Urchin egg	Fly egg	Zebrafish egg
Diameter (μm)	10	100	175 × 500	700
Volume (l)	0.5×10^{-12}	0.5×10^{-9}	10×10^{-9}	180×10^{-9}
Surface (mm^2)	0.3×10^{-3}	0.03	0.22	1.54
V/S (l/mm^2)[b]	1.7×10^{-9}	17×10^{-9}	45×10^{-9}	117×10^{-9}
Normalized V/S[c]	0.015	0.15	0.38	1.0
100% Conversion (days)[d]	0.7	7	19	50

[a]The size of a single tissue culture cell was compared with various egg sizes.
[b]The 'volume/surface' ratio (V/S) of various cells.
[c]The 'volume/surface' ratio was normalized in the *Drosophila* ovary, in which 0.33% of the apo-aequorin was converted after a 4 h incubation.
[d]The 100% conversion times were extrapolated from apo-aequorin conversion rates measured in *Drosophila*. Calculations are based on an apo-aequorin concentration of 1 μM in each of the cell types and assume that the 100% conversion time is proportional to the 'volume/surface' ratio; see text for details.

only 0.33% of the aequorin within an *Drosophila* ovary is converted in 4 h, it would take 50 days (!) to get 100% conversion. In calculating the 100% conversion time, we assume that coelenterazine uptake is a linear process. This assumption was made on the basis that once the coelenterazine is inside the cell, it will bind to the apo-aequorin almost immediately (in vitro reconstitution only takes 11 min at 24°C [3]). Once bound, the coelenterazine will not be free to diffuse back into the medium and it can thus be expected that coelenterazine uptake continues at the same rate until all apo-aequorin is bound.

We propose that the uptake of coelenterazine depends on the surface of a specimen, i.e. a bigger surface would allow a faster uptake. However, bigger eggs will also need more coelenterazine uptake (assuming all eggs express the same concentration of apo-aequorin). We thus hypothesize that the 100% conversion time is proportional to the 'volume/surface' ratio. In round eggs, this ratio is proportional to the radius of the egg. For example, a sea urchin egg which is 7 times smaller than the 700 μm ovary, would have a 7 times shorter conversion time (100% conversion in 7 days). Even smaller cells such as a single *Dictyostelium* cell (~70 times smaller than the ovary) would attain 100% conversion in only 18 h. This 18 h extrapolation fits well with the reconstitution rates measured in cultured *Dictyostelium* cells. These small cells were shown to attain optimal reconstitution of intracellular aequorin in 24 h [9]. The volumes, surfaces and extrapolated reconstitution rates of different eggs and cells are summarized in the Table.

CONCLUSIONS

At least 20 femtomoles of recombinant aequorin is needed for imaging resting levels of free cytosolic calcium. This corresponds to aequorin concentrations of 0.11 μM in zebrafish eggs, 2 μM in *Drosophila* eggs, 40 μM in sea urchin eggs. Reconstitution experiments in *Drosophila* suggest that aequorin formation is limited by coelenterazine uptake. Based on the rate of coelenterazine uptake in *Drosophila*, we estimate that complete conversion of 1 μM apo-aequorin would take 50 days in zebrafish eggs, 19 days in *Drosophila* eggs, 7 days in sea urchin eggs or 18 h in a 10 μm tissue culture cell. The present study suggests that work based upon genetically introduced apo-aequorin will be most successful when large amounts of small cells can be incubated in coelenterazine. For work during embryonic development this would involve introducing coelenterazine into the blood stream of late stage embryos. Imaging early stage embryos will still require injection of aequorin.

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