

Transient expression of apoaequorin in zebrafish embryos: extending the ability to image calcium transients during later stages of development

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ABSTRACT When aequorin is microinjected into cleavage-stage zebrafish embryos, it is largely used up by ~24 hours. Thus, it is currently not possible to image Ca²⁺ signals from later stages of zebrafish development using this approach. We have, therefore, developed protocols to express apoaequorin, i.e., the protein component of aequorin, transiently in zebrafish embryos and then reconstitute intact aequorin *in vivo* by loading the coelenterazine co-factor into the embryos separately. Two types of apoaequorin mRNA, *aeq*-mRNA and *aeq::EGFP*-mRNA, the latter containing the enhanced green fluorescent protein (EGFP) sequence, were *in vitro* transcribed and when these were microinjected into embryos, they successfully translated apoaequorin and a fusion protein of apoaequorin and EGFP (apoaequorin-EGFP), respectively. We show that *aeq::EGFP*-mRNA was more toxic to embryos than equivalent amounts of *aeq*-mRNA. In addition, in an *in vitro* reconstitution assay, apoaequorin-EGFP produced less luminescence than apoaequorin, after reconstitution with coelenterazine and with the addition of Ca²⁺. Furthermore, when imaging intact coelenterazine-loaded embryos that expressed apoaequorin, Ca²⁺ signals from ~2.5 to 48 hpf were observed, with the spatio-temporal pattern of these signals up to 24 hpf, being comparable to that observed with aequorin. This transient aequorin expression approach using *aeq*-mRNA provides a valuable tool for monitoring Ca²⁺ signaling during the 24–48 hpf period of zebrafish development. Thus, it effectively extends the aequorin-based Ca²⁺ imaging window by an additional 24 hours.

KEY WORDS: *aequorin*, *apoaequorin*, *coelenterazine*, Ca²⁺, *zebrafish*

Introduction

Calcium ions (Ca²⁺) are ubiquitous second messengers that are known to control a wide variety of biological processes, such as gene transcription, cytoskeletal remodeling, membrane trafficking, cytokinesis and cell movement (reviewed by Berridge *et al.*, 2003). Via combinations of these processes it has also long been suggested that Ca²⁺ signaling, in the form of pulses, waves and steady gradients, might play a key role in pattern formation during embryogenesis, from the earliest stages of development to the differentiation of specific cell types (reviewed by Webb and Miller, 2003). Whereas the intracellular Ca²⁺ concentration is relatively low in resting cells (i.e., ~60-100 nM), it can increase to ~1 μM or more, in a short period of time when cells are stimulated to perform particular functions (Bootman *et al.*, 2001). Thus, by changing the dimensions of cytosolic Ca²⁺ events in terms of space, time and amplitude, information can be rapidly and efficiently exchanged

both across and between cells (Berridge *et al.*, 2003).

One of the first reporters used to monitor intracellular Ca²⁺ levels in living cells was the bioluminescent Ca²⁺-sensitive protein complex, aequorin (Ridgway and Ashley, 1967). Originally isolated from the luminescent jellyfish *Aequorea aequorea* (Shimomura *et al.*, 1962), the aequorin complex is made up from an apo-protein (apoaequorin) of ~21 kDa that contains four helix-loop-helix "EF-hand" domains (three of which can bind to Ca²⁺; Head *et al.*, 2000), a prosthetic co-factor of ~420 Da (coelenterazine) and O₂, in the form of peroxide (Shimomura and Johnson, 1978). In the presence of free Ca²⁺, the coelenterazine is oxidized to coelenteramide, with an associated release of CO₂ and blue light (at ~470 nm) is produced (Shimomura and Johnson, 1973). Following this reaction, the aequorin complex is effectively "spent" and active aequorin

Abbreviations used in this paper: Ca²⁺, calcium ions; EGFP, enhanced green fluorescent protein; hpf, hours post-fertilization; RLU, relative light units

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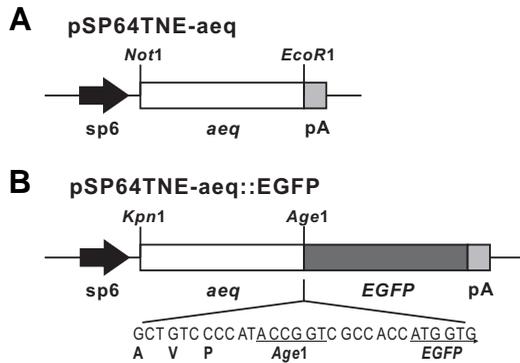


Fig. 1. Map of the pSP64TNE-aeq and pSP64TNE-aeq::EGFP plasmids. **(A)** For pSP64TNE-aeq, the full length sequence of aeq (591 bp) was cloned in the pSP64TNE plasmid, while **(B)** for pSP64TNE-aeq::EGFP, the aeq sequence, minus its stop codon was ligated in-frame to the 5' end of EGFP to make a fusion protein of apoaequorin and EGFP in pSP64TNE. Black line, pSP64TNE plasmid vector sequences; sp6 (black arrow), sp6 promoter sequence; aeq, apoaequorin cDNA sequence; EGFP, enhanced green fluorescent protein sequence; pA, polyadenylation signal.

cannot be regenerated unless additional coelenterazine is provided (Shimomura and Johnson, 1975). In living cells, aequorin has a wide dynamic range (i.e., 0.1–100 μM Ca^{2+}), where the light output of the aequorin luminescence reaction is approximately proportional to the square of the free Ca^{2+} concentration and thus it shows inherent contrast enhancement (Shimomura and Inouye, 1996). In addition, Ca^{2+} -bound aequorin emits light without excitation, thus the autofluorescence and photo-induced cytotoxicity that can take place with fluorescent Ca^{2+} reporters, does not occur with aequorin. Aequorin has also been shown not to interfere with the normal development of living embryos (Miller *et al.*, 1994). This allows for long-term Ca^{2+} imaging, which is particularly suited for studying

embryonic development (Miller *et al.*, 1994; Ashworth and Brennan, 2005).

One of the current popular vertebrate models for studying embryogenesis in recent years is the zebrafish (*Danio rerio*). A combination of the optical transparency, large size and rapid development of their embryos makes them particularly ideal for Ca^{2+} imaging. Through the microinjection of aequorin into the blastoderm of zebrafish embryos at the single-cell stage and the use of a custom-designed Photon Imaging Microscope System (PIMS), we have been able to continuously image the Ca^{2+} signals from zebrafish embryos, beginning at egg activation (Lee *et al.*, 1999), then through ooplasmic segregation and cytokinesis in the early Cleavage Period (Webb *et al.*, 1997; Leung *et al.*, 1998), during the Gastrula (Gilland *et al.*, 1999) and Segmentation (Webb and Miller, 2003) Periods and up to around the prim-6 stage around the start of the Pharyngula Period. Up until now, however, we have not been able to image Ca^{2+} signals in embryos at later stages of development. This is because aequorin that is injected into embryos at the one-cell stage and becomes partitioned into daughter cells by subsequent cell divisions, is used up by ~24 hours. It is also clearly impractical to microinject the reporter into each of the tens of thousands of cells that comprise these later-stage embryos. One way to get around this restriction is to express the protein component of aequorin (i.e., apoaequorin), transgenically in zebrafish and then to reconstitute the active protein complex *in vivo* by loading the coelenterazine co-factor into the embryos separately. The techniques used for transforming apoaequorin cDNA into single cell organisms (e.g. *Escherichia coli*; Inouye *et al.*, 1989) and some simple multicellular organisms (e.g. the slime mold, *Dictyostelium discoideum*, Saran *et al.*, 1994), as well as in various mammalian tissue culture cells (Button and Brownstein, 1993), have already been successfully developed. Active aequorin has then been reconstituted within these transgenic cells, tissues or whole organisms via a very straightforward incubation in coelenterazine solution. To date, however, there is just a single

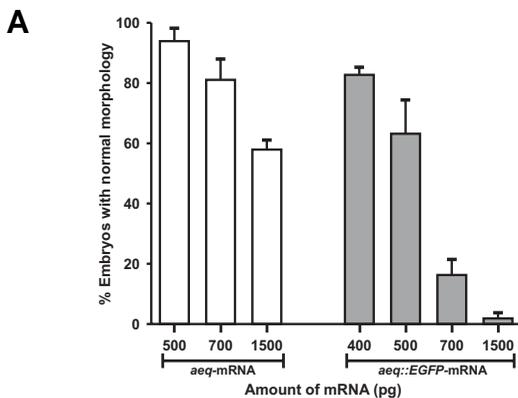
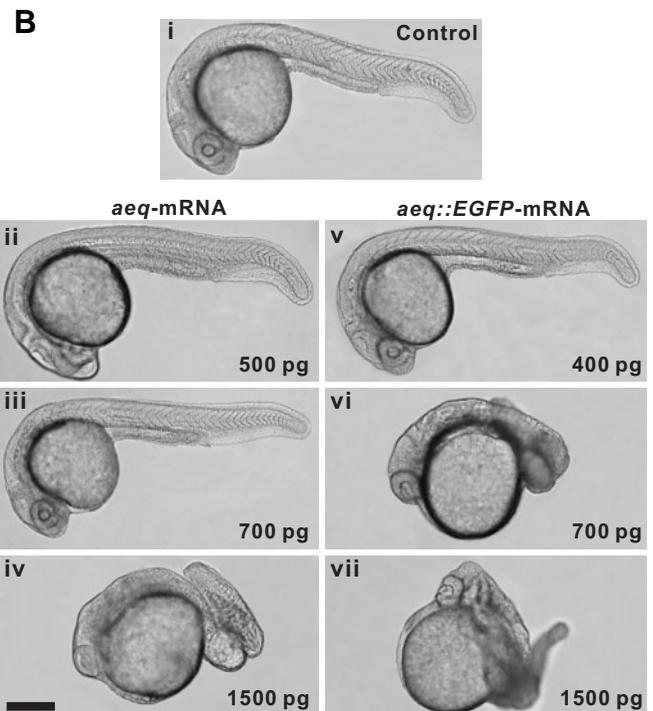


Fig. 2. Effect of aeq-mRNA and aeq::EGFP-mRNA on normal development. Embryos were injected with different amounts of aeq-mRNA or aeq::EGFP-mRNA at the 1-cell stage and then observed after 24 h. **(A)** Bar graph to illustrate the percentage of normal embryos that developed for each amount of mRNA injected. The data are expressed as mean \pm SEM ($n \geq 3$). **(B)** Bright-field images to show the effect of injecting different amounts of aeq-mRNA and aeq::EGFP-mRNA into embryos compared to (i) an uninjected control embryo at 24 hpf. Representative examples of embryos injected with (ii) 500 pg, (iii) 700 pg or (iv) 1500 pg aeq-mRNA, or with (v) 400 pg, (vi) 700 pg or (vii) 1500 pg aeq::EGFP-mRNA. Embryos injected with >700 pg aeq-mRNA or >400 pg aeq::EGFP-mRNA exhibited defects in the development of both head and tail. Scale bar, 200 μm .



recent report that describes the injection of cDNA and also mRNA apoaequorin constructs into zebrafish embryos (Ashworth and Brennan, 2005). The authors also highlight the challenges in detecting Ca^{2+} signals in the apoaequorin expressing fish and discuss some of the problems in loading coelenterazine into later-stage embryos for the generation of active aequorin.

In this study, therefore, we expand on the work reported by Ashworth and Brennan (2005) and describe in detail, protocols developed to express apoaequorin transiently and ubiquitously in zebrafish embryos, via the injection of apoaequorin mRNA followed by incubation with coelenterazine to reconstitute active aequorin *in vivo*. We demonstrate that Ca^{2+} signals can be observed from ~2.5 to 48 hpf and we show that the spatio-temporal pattern of these signals up to 24 hpf is comparable to that observed through injecting single cell-stage embryos with recombinant aequorin. The advantage of this new technique, however, is the additional time (i.e., from 24 to 48 hpf) that it extends the non-invasive imaging window. We suggest, therefore, that it will be a useful interim methodology for Ca^{2+} imaging until such time as stable transgenic lines of apoaequorin-expressing fish are developed. The extension of the aequorin-based imaging period through the Pharyngula Period (24 to 48 hpf) to the beginning of the Hatching Period (48 to 72 hpf) is significant, as it will thus encompass the important developmental events of the former. These include the appearance of tactile sensitivity; the coordination of myotome contraction resulting in rhythmic bouts of swimming; the development of the circulatory system and the initiation of the heart-beat (Kimmel *et al.*, 1995). These events may require different forms of Ca^{2+} signaling, both for their development and their function. We propose, therefore, that a transient apoaequorin expressing zebrafish may provide a first step in investigating possible Ca^{2+} related signaling pathways and their down-stream targets.

Results and Discussion

In this study, two types of apoaequorin mRNA, i.e., *aeq*-mRNA and *aeq::EGFP*-mRNA, were *in vitro* transcribed using the corresponding pSP64TNE-*aeq* and pSP64TNE-*aeq::EGFP* plasmids. On their subsequent microinjection into 1-cell stage embryos, they were successfully translated into apoaequorin and an apoaequorin-EGFP fusion protein, respectively.

Testing mRNA toxicity

It has been reported that injection of high concentrations of mRNA can lead to abnormal development and may indeed be lethal to zebrafish embryos (Gilmour *et al.*, 2002). Thus, the maximum amount of *aeq*-mRNA and *aeq::EGFP*-mRNA that could be loaded into zebrafish embryos and still result in normal development was determined. Embryos were injected into the blastodisc at the 1-cell stage with increasing amounts of these mRNAs, after which they were examined for normal development (i.e., by comparison with uninjected control embryos), at 24 hpf (see Fig. 2). Fig. 2A shows that as much as 700 pg *aeq*-mRNA could be injected into embryos and more than 80% (i.e., $81.0 \pm 7.0\%$) developed normally, when examined at 24 hpf. Indeed, when injected with 500 pg *aeq*-mRNA, ~94.0 ± 4.0% embryos were normal after 24 hours. When the amount of mRNA was increased to 1500 pg per embryo, however, the number of normal embryos observed decreased to

~60%. The *aeq::EGFP*-mRNA also showed a dose-dependent increase in toxicity in embryos, however, >80% normal development (i.e., $83.0 \pm 2.5\%$) could only be achieved when embryos were injected with just 400 pg mRNA. At 500 pg *aeq::EGFP*-mRNA, the number of normal embryos after 24 hours was just $63 \pm 11\%$ and amounts of 700 pg and 1500 pg produced only $16 \pm 5\%$ and $2 \pm 2\%$ normal embryos, respectively.

The effect of injecting increasing amounts of *aeq*-mRNA and *aeq::EGFP*-mRNA on development is also shown in the bright-field images in Fig. 2B. When embryos were injected with 700 pg *aeq*-mRNA or 400 pg *aeq::EGFP*-mRNA, their gross morphology after 24 hours was comparable to the uninjected controls. However, when embryos were injected with more than either 700 pg *aeq*-mRNA or 400 pg *aeq::EGFP*-mRNA, a range of abnormalities was observed. These included defects in the development of the head and tail. The reasons for mRNA-induced abnormal development or death are thought to be both varied and complex, but are suggested to be at least in part, due to the extra demand on the protein-making resources of the embryo (Gilmour *et al.*, 2002). In subsequent experiments, 700 pg *aeq*-mRNA and 400 pg *aeq::EGFP*-mRNA were used.

Expression of EGFP in the *aeq::EGFP*-mRNA injected embryo

The *aeq::EGFP*-mRNA was specifically prepared in order to test whether the expression of EGFP could be used to provide spatio-

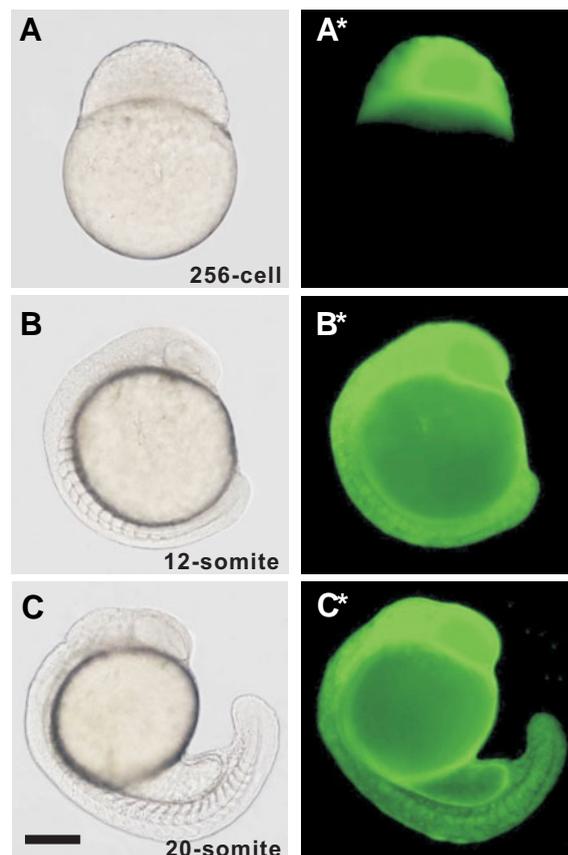


Fig. 3. Expression of enhanced green fluorescent protein (EGFP) in the *aeq::EGFP*-mRNA injected embryos. (A,B,C) Bright-field and (A*,B*,C*) respective EGFP fluorescence images of a representative *aeq::EGFP*-mRNA injected embryo at the (A, A*) 256-cell, (B, B*) 12-somite and (C, C*) 20-somite stages. Scale bar, 200 μ m.

temporal information about the transiently expressed apoaequorin in the developing embryo. GFP has been successfully tagged to other proteins in a variety of organisms, including zebrafish (reviewed by March *et al.*, 2003). Thus, embryos were injected with 400 pg *aeq::EGFP*-mRNA into the blastodisc at the 1-cell stage and fluorescence images were acquired at different stages of development. Figure 3 illustrates the pattern and level of expression of EGFP in a representative *aeq::EGFP*-mRNA injected embryo at the 256-cell (~2.5 hpf), 12-somite (~15 hpf) and 20-somite stages (~19 hpf). An intense and ubiquitous pattern of EGFP fluorescence is observed at all the stages shown. This result is not unexpected, having been reported previously for a similar GFP-apoaequorin mRNA-based probe (Ashworth and Brennan, 2005).

Time course of apoaequorin expression and estimation of expression levels

Embryos were injected with either 700 pg *aeq*-mRNA or 400 pg *aeq::EGFP*-mRNA into the blastodisc at the 1-cell stage and Western blot analysis was conducted to estimate the level of apoaequorin expression at different time points up to 30 hpf (Fig. 4). Representative examples ($n=3$) of the time course of apoaequorin expression following *aeq*-mRNA or *aeq::EGFP*-mRNA injection are shown in Fig. 4B and 4C, respectively. These expression levels were quantified and then normalized using recombinant aequorin as an internal positive control, before graph plotting.

The graph (Fig. 4A) demonstrates that when embryos were injected with either *aeq*-mRNA or *aeq::EGFP*-mRNA, the expression of apoaequorin could be detected as early as 2 hpf and

reached a peak level at 4 hpf, after which it started to decline. This drop in the level of apoaequorin expression was more rapid in the *aeq*-mRNA-injected embryos. At 30 hpf, for example, no apoaequorin was detected in the *aeq*-mRNA injected embryos, whereas it was still detected at nearly half the maximum level in the *aeq::EGFP*-mRNA injected embryos at this time. The lower level of apoaequorin expression that is shown in the graph (Fig. 4A) for the *aeq::EGFP*-mRNA injected embryos, might simply be due to the lower amount of mRNA injected (i.e., 400 pg per embryo, as opposed to 700 pg).

In order to estimate the concentrations of apoaequorin and apoaequorin-EGFP protein expressed, the bands from three separate Western blot experiments for each expression vector (including those shown in Fig. 4 B,C) were quantified by comparing them to an internal positive control lane loaded with 20 pg of aequorin. The mean values obtained from three separate experiments indicated that approximately 83.3 pg of apoaequorin and 34.2 pg of apoaequorin-EGFP were expressed in embryos injected with *aeq*-mRNA or *aeq::EGFP*-mRNA, respectively. Assuming the volume of a zebrafish embryo is ~128 nl and approximately 68% of this is water (Leung *et al.*, 1998), at 4 hpf this equates to around 45.6 nM of apoaequorin and 18.7 nM of apoaequorin-EGFP, respectively.

Thus, the results of these experiments indicate that even though *aeq::EGFP*-mRNA was more toxic to embryos than equivalent amounts of *aeq*-mRNA, both mRNAs were successful in translating apoaequorin and apoaequorin-EGFP, respectively in embryos. We thus proceeded to determine whether these proteins had luminescent capability on reconstitution with *f*-coelenterazine followed by the addition of Ca^{2+} .

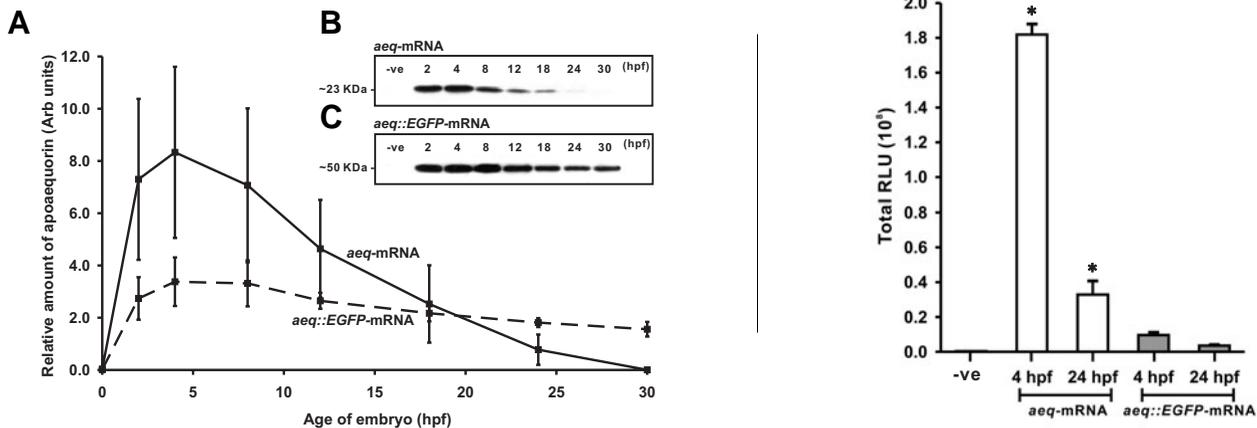


Fig. 4 (Left column). Apoaequorin expression profiles of *aeq*-mRNA and *aeq::EGFP*-mRNA injected embryos. A time-course Western blot analysis was conducted using embryos that had been injected with either *aeq*-mRNA or *aeq::EGFP*-mRNA at the single cell stage and then embryo extract prepared at 2, 4, 8, 12, 18, 24 and 30 hpf. (A) The line graph illustrates the mean \pm SEM ($n=3$) level of apoaequorin expression in extract from embryos injected with 700 pg *aeq*-mRNA or 400 pg *aeq::EGFP*-mRNA, relative to the internal control, aequorin. (B,C) Representative examples of Western blots to show the relative level and temporal expression pattern of apoaequorin in extract that was prepared from embryos injected with (B) *aeq*-mRNA or (C) *aeq::EGFP*-mRNA. Extract prepared from 2 embryos was loaded into each lane.

Fig. 5 (Right column). Comparison of the total luminescence from embryo extract, prepared from embryos that had been injected with 700 pg *aeq*-mRNA or 400 pg *aeq::EGFP*-mRNA at the 1-cell stage. Aequorin was reconstituted from apoaequorin in the extract by incubation with *f*-coelenterazine, after which $CaCl_2$ was added at 4 hpf and 24 hpf and the resulting luminescence quantified. The data are expressed as mean \pm SEM total relative light units (RLU) of 3 independent experiments. The RLU from each experiment represents the total luminescence produced from an extract obtained from 10 mRNA-injected embryos in 1 min immediately after the addition of Ca^{2+} . * Indicates measurements that are statistically significant at $P<0.01$ using one-way ANOVA (parametric) and Newman-Keuls multiple comparison test.

Comparative *in vitro* aequorin reconstitution assay of extract prepared from *aeq*-mRNA injected embryos

The amount of luminescence produced by embryo extract, which had been prepared from embryos that had been injected with 700 pg *aeq*-mRNA or 400 pg *aeq::EGFP*-mRNA was compared (Fig. 5). The amount of luminescence produced by embryos that were not injected with mRNA but that had been treated with *f*-coelenterazine at 4 hpf is shown in lane 1 (labeled '-ve') as a negative control. Figure 5 shows that the level of luminescence produced at both 4 hpf and 24 hpf in *aeq*-mRNA injected embryos was significantly greater than that produced at the same time points in the *aeq::EGFP*-mRNA injected embryos, respectively. For example, at 4 hpf, the luminescence produced from the *aeq*-mRNA injected embryos was ~18-fold higher than that produced by the *aeq::EGFP*-mRNA injected embryos at the same time (i.e., $1.82 \pm 0.06 \times 10^8$ RLU compared to $0.1 \pm 0.02 \times 10^8$ RLU). In addition, the luminescence produced from *aeq*-mRNA injected embryos at 4 hpf was ~5.5-fold higher than that from *aeq*-mRNA injected embryos at 24 hpf (i.e., $1.82 \pm 0.06 \times 10^8$ RLU compared to $0.33 \pm 0.08 \times 10^8$ RLU). RLU values represent the amount of light collected for 1 min immediately after the addition of 200 μ l of 150 mM Ca^{2+} . *In vitro* control experiments indicated that 1 ng of recombinant *f*aequorin produces $\sim 3.92 \times 10^8$ RLU (data not shown as it would over-extend the scale on the y-axis of Fig. 5). Using this number, we can estimate the approximate amounts of *f*aequorin and *f*aequorin-EGFP reconstituted at various times from their respective RLU outputs. These comparisons suggest that ~463.1 pg of *f*aequorin and ~23.7 pg of *f*aequorin-EGFP were reconstituted at 4 hpf in *aeq*-mRNA and *aeq::EGFP*-mRNA injected embryos, respectively. Using the same assumptions for embryo volume and water content indicated earlier (Leung *et al.*, 1998) this indicates a final concentration of functional *f*aequorin of ~254 nM and *f*aequorin-EGFP of ~13 nM at 4 hpf. Our estimate for *in vitro* *f*aequorin reconstitution is thus similar to the 110 nM suggested previously, to be required to image resting levels of Ca^{2+} in zebrafish embryos (Créton *et al.*, 1997). However, the luminescent-based value for reconstituted *f*aequorin is ~5-fold higher than that estimated for apoaequorin protein expression derived from the Western blot protein quantification. On the other hand, the comparative values for apoaequorin-GFP expression and *f*apoaequorin-EGFP reconstitution are somewhat similar (i.e., comparing 34.25 pg with 23.7 pg, respectively). Along with the inherent inaccuracies that accompany these quantification techniques, one additional reason for this large difference with regards to comparing apoaequorin expression with *f*aequorin reconstitution might be attributed to the protein stabilization effect conferred by the covalent bonding of apoaequorin to *f*-coelenterazine. In the case of apoaequorin-EGFP, the fusion of the EGFP portion might in itself confer additional resistance to protein degradation and as a result, there is little difference between the values obtained via these two techniques.

We suggest that the low luminescent ability of the apoaequorin-EGFP might be due to the fact that in the preparation of the pSP64TNE-*aeq::EGFP* plasmid, the EGFP sequence was linked to the C-terminus of the apoaequorin sequence. As the apoaequorin C-terminal tail is known to play a key role in binding coelenterazine, the addition of the EGFP sequence in this region might result in a reduced level of coelenterazine binding (Head *et al.*, 2000). We suggest that linking the EGFP sequence to the N-terminus rather

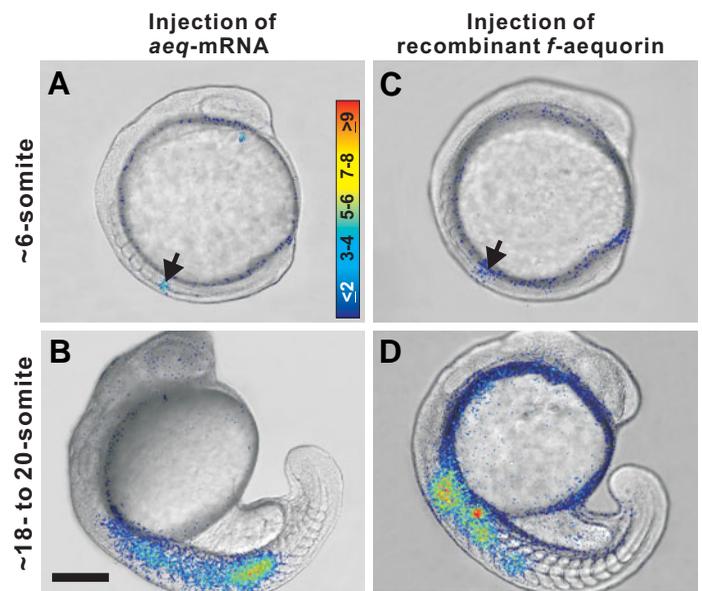


Fig. 6. A comparison of the intracellular Ca^{2+} signaling patterns observed in representative (A,B) *aeq*-mRNA injected embryos and (C,D) *f*-aequorin injected embryos during the Segmentation Period.

(A,B) Embryos were injected with *aeq*-mRNA at the single-cell stage and then bathed with *f*-coelenterazine to reconstitute aequorin from apoaequorin. (C,D) Embryos were injected into the top of the yolk with *f*-aequorin during the Cleavage Period. (A,C) are trunk signals observed at ~6-somite stage, during early segmentation, while (B,D) are trunk signals that occur during later segmentation, at ~18 to 20-somite stage. Each panel represents 60 sec of accumulated light. Color scale indicates luminescent flux in photons/pixel. Scale bar, 200 μ m.

than the C-terminus of apoaequorin might improve the luminescence ability. Indeed, Baubet *et al.* (2000) have shown that this type of plasmid design (for a fusion protein consisting of GFP and apoaequorin) works well in mammalian cells.

When we introduce recombinant *f*aequorin into single cell-stage zebrafish embryos, our normal procedure is to inject approximately 3.5 nl of a 1% *f*aequorin solution (Webb *et al.*, 1997). This represents a final concentration of *f*aequorin within the embryo of approximately 18 μ M. This is approximately 70-fold more than the final concentration calculated at the 4 hpf expression and reconstitution peak (i.e., ~254 nM). Créton *et al.*, however, have reported that the estimated *in vivo* half-life of *f*-aequorin (a semi-synthetic aequorin similar to *f*aequorin; Shimomura *et al.*, 1989) is only around 3 hours (Créton *et al.*, 1997). In addition, it is clear from Fig. 8 that although injection of recombinant *f*aequorin may initially load an embryo with a substantially greater amount of aequorin, for longer term imaging (i.e., post 24 hpf) transiently expressed apoaequorin recombined with *f*-coelenterazine does offer a significant advantage.

In vivo reconstitution of aequorin in *aeq*-mRNA injected intact zebrafish embryos

Because of the reduced luminescence output of apoaequorin-EGFP, the *aeq*-mRNA injected embryos alone were used for the *in vivo* reconstitution experiment. Thus, dechorionated embryos that had been injected with 700 pg *aeq*-mRNA at the 1-cell stage, were incubated in *f*-coelenterazine at the 64-cell stage (i.e., 2 hpf)

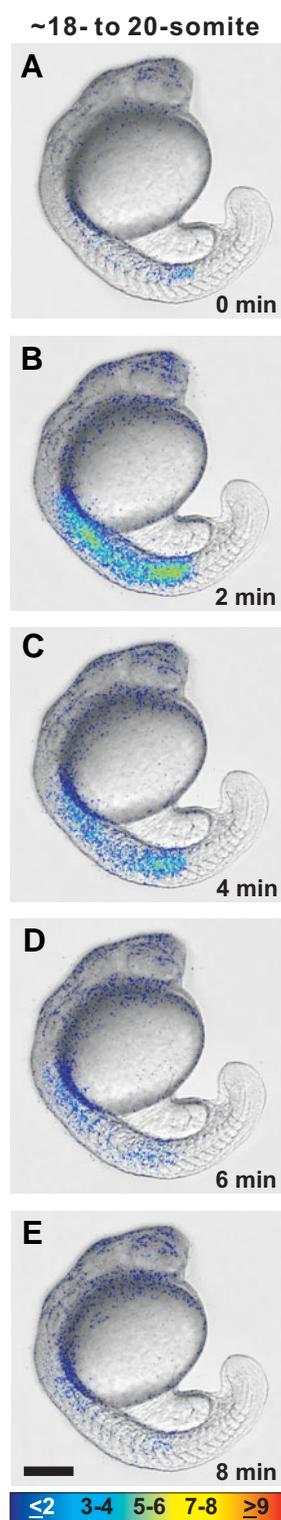


Fig. 7. The localized intercellular Ca^{2+} signals that are generated in the trunk of the representative embryo in Fig. 6B are shown in greater detail. This embryo had been injected with *aeq*-mRNA at the single-cell stage and then bathed with *f*-coelenterazine to reconstitute aequorin from apoaequorin. Each panel represents 60 sec of aequorin generated light with a 120 s gap between each image. Color scale indicates luminescent flux in photons/pixel. Scale bar, 200 μm .

and then the spatial and temporal pattern of Ca^{2+} signals (as determined by changes in luminescence) produced by these embryos was recorded using our PIMS (Fig. 6). The 64-cell stage was chosen as the starting point for the coelenterazine incubation both because (i) the embryos had started to express apoaequorin (Fig. 4) and (ii) the diffusion of coelenterazine into the embryo was considered to be still relatively efficient as a significant number of blastomeres were still in contact with the bathing medium at this stage. The incubation of embryos at earlier stages was not thought to be of any advantage as apoaequorin was not being expressed and so the potential for coelenterazine to be exposed to oxidizing agents (prior to being protected through binding with apoaequorin; Shimomura, 1995; Head *et al.*, 2000), was considered to be greater. The coelenterazine analogue, *f*-coelenterazine was chosen specifically as it has the same half-life as *native* coelenterazine, however, once it is reconstituted into the aequorin complex, it produces almost 20-fold higher luminescence in the presence of Ca^{2+} (Shimomura, 1991). Furthermore, *f*-coelenterazine has been reported to have the highest permeability in cells (Shimomura, 1997).

When *aeq*-mRNA injected embryos were imaged with our PIMS, Ca^{2+} signals were first detected at ~ 2.5 hpf, after approximately 30-60 minutes incubation in coelenterazine solution (data not shown) and they were observed for up to 48 hours. This clearly demonstrates that transiently expressed apoaequorin can successfully be reconstituted to aequorin in zebrafish embryos *in vivo*. Furthermore, the Ca^{2+} signals observed from the 256-cell stage (~ 2.5 hpf) to

the prim-5 stage (~ 48 hpf) in *aeq*-mRNA injected embryos were similar to those observed when *f*-aequorin had been microinjected into early stage embryos. Figure 6 shows examples of what appears to be two different classes of Ca^{2+} signals generated in the trunk of embryos that could be detected and distinguished from each other, in representative *aeq*-mRNA injected (Fig. 6 A,B) and recombinant *f*-aequorin (Fig. 6 C,D) injected embryos at both the 6-somite and 18-somite stages, respectively. At the 6-somite stage (Fig. 6 A,C), a highly localized Ca^{2+} transient with a duration between 60 to 120 seconds occurs in the trunk ($n = 9$), the temporal and spatial characteristics of which appear to correlate with the formation of the next somite. Similar somitogenic Ca^{2+} signals have been previously reported during the Segmentation Period in aequorin-injected zebrafish (Créton *et al.*, 1998). On the other hand, at the 18-20-somite stage (Fig. 6 B,D), a more widespread, longer lasting (i.e., ~ 10 min) Ca^{2+} transient was observed, generated by the ~ 12 -14 anterior-most somites alone (i.e., absent from the 6 posterior-most somites). The Ca^{2+} signal in the trunk of the *aeq*-mRNA-injected embryo at the 18-20-somite stage is shown in more detail in Fig. 7 and clearly indicates that there is sufficient aequorin reconstituted in the cells of the trunk to sustain a Ca^{2+} transient that lasts for at least 10 minutes. This trunk signal was seen in a consistent and reliable manner ($n = 10$) and the embryos generating them developed normally. The developmental and physiological functions of this later signal have yet to be determined. It has been reported, however, that at around this developmental stage the first muscle contractions begin in individual myotomes (Kimmel *et al.*, 1995; Saint-Amant and Drapeau, 1998). The duration of this signal clearly indicates that it is not likely to be associated with a single muscle twitch. The onset of these contractions, however, has been reported to be correlated with axonal ingrowth into the developing muscle from the first primary motorneurons (Myers *et al.*, 1986). Thus, this sustained trunk Ca^{2+} transient may play a role in some as yet poorly understood aspect of muscle development that occurs over an equivalent time frame.

Figure 8A illustrates the Ca^{2+} signals observed in *aeq*-mRNA- and recombinant *f*-aequorin-injected embryos from 27-36 hpf. The level of luminescence produced by the representative *f*-aequorin-injected embryo was minimal (i.e., running at around 4-5 photons/sec for much of the 6-h imaging period) and failed to register any Ca^{2+} spiking activity. This represents $\sim 0.02\%$ of the total amount of luminescence emitted during the entire imaging window. On the other hand, the *aeq*-mRNA-injected embryo exhibited both a far greater overall level of luminescence (i.e., approximately 75-100 photons/sec) as well as a more complex pattern of Ca^{2+} spiking. When visualized, each individual spike represents a rapid Ca^{2+} transient lasting in the order of a few seconds only. We suggest that this series of Ca^{2+} spikes represent a third class of trunk-generated Ca^{2+} transients and may result from the more rapid contractions that normally result from touch-based responses during this period of zebrafish development (Saint-Amant and Drapeau, 1998). Figure 8B illustrates a representative example ($n=5$) of another form of trunk Ca^{2+} transient that was generated at ~ 47 hpf. These transients had a duration of ~ 60 sec and would thus appear to be of a different class again to those illustrated in Figs. 7,8A. They appear to be somewhat similar in duration to the ~ 60 to 120-sec duration Ca^{2+} transients that have been reported to be generated in intact

myotome isolated from stage 23/24 (i.e., 25 to 26 hpf) *Xenopus* embryos and shown to be essential for myofibril organization and the formation of sarcomeres (Ferrari and Spitzer, 1999).

Although the precise developmental or physiological function of the trunk-generated Ca^{2+} transients illustrated in Figs. 6 to 8 are far from certain, they clearly indicate the potential of transiently expressing apoaequorin in zebrafish as an *in situ* Ca^{2+} imaging tool.

Conclusions

As shown by the time-course Western blot analysis (see Fig. 4), the expression of apoaequorin in the *aeq*-mRNA injected embryos ceased after 24 hours. This would suggest that there is a definitive limit to the amount of apoaequorin protein made in these embryos. However, experiments like the one illustrated in Fig. 8B indicate that Ca^{2+} signals can still be detected up to ~48 hpf. This may be due to (1) a stable aequorin complex being formed, which is more resistant to the action of cellular proteases, once *f*-coelenterazine binds to apoaequorin (Shimomura, 1991) and (2) the fact that the resting level of Ca^{2+} within quiescent cells of a zebrafish embryo have been reported to be low, i.e., around 60 nM (Créton *et al.*, 1998), resulting in the finite quantities of reconstituted aequorin being used up at a very low rate (i.e., when cells are not generating Ca^{2+} signals).

There would appear to be two possible drawbacks to using the transient expression approach. Firstly, it cannot be used for imaging Ca^{2+} in embryos at stages before the apoaequorin protein is expressed in sufficient quantities to allow for detectable signal after reconstitution (i.e., before ~2.5 hpf, the 256-cell stage). This problem can be overcome by simply injecting aequorin at the one cell stage. The second drawback results from the ubiquitous nature of mRNA-derived expression. Between 2.5 and 48 hpf (i.e., the imaging window of this technique) the three-dimensional complexity of the embryo increases dramatically, especially following gastrulation. Thus, it will become more challenging to determine precisely which group of cells, tissue or organ anlage might be responsible for generating a particular signal in later stage embryos. We plan to resolve this shortcoming in the future by developing stable lines of transgenic zebrafish that both express apoaequorin in a tissue-specific manner and further extend the Ca^{2+} -imaging window.

Nevertheless, we believe that the transient expression approach is a valuable interim tool (that can be used while the transgenic lines are being generated), for monitoring Ca^{2+}

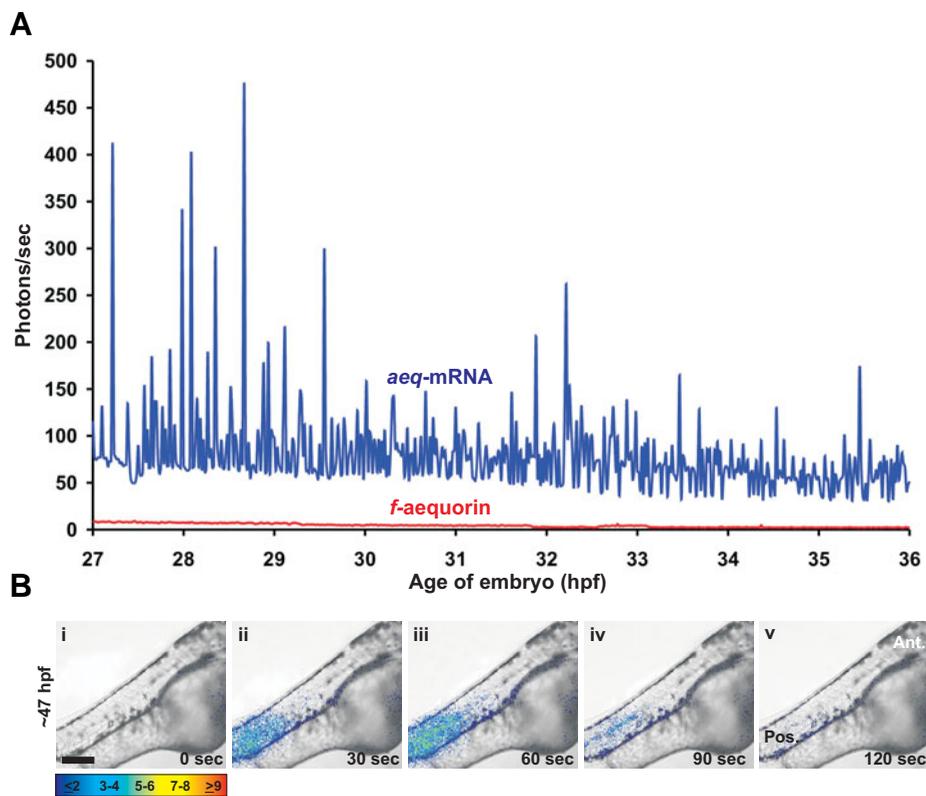


Fig. 8. The use of *aeq*-mRNA extends the aequorin-based Ca^{2+} imaging window beyond 24 hpf. (A) A comparison of the aequorin-generated luminescence observed in representative *aeq*-mRNA- and *f*-aequorin-injected embryos from 27–36 hpf. Traces were generated from data points representing 60 sec of luminescence that were acquired every 60 s for an imaging field covering approximately 64,500 pixels. **(B)** An example of a localized intercellular Ca^{2+} signal that is generated in the trunk at ~47 hpf. The panels show the representative pattern of luminescence superimposed on corresponding bright-field images. Each panel displays 60 s of accumulated light with a 30 s interval between each image. Ant. and Pos. represent anterior and posterior, respectively. Color scale indicates luminescent flux in photons/pixel. Scale bar, 200 μ m.

signaling events that occur during the relative later stages of zebrafish development (i.e., 24 to 48 hpf), when injected aequorin has been used up. This effectively extends the aequorin-based Ca^{2+} imaging period by an additional 24 hours. Thus, in combination with injecting aequorin at the single-cell stage, Ca^{2+} signaling in zebrafish can be now continuously monitored during development from fertilization up to the long pec stage, at 48 hpf. We suggest, therefore, that this method represents a significant step forward in the aequorin-based imaging technique by providing a straightforward and rapid first step for the study of developmental Ca^{2+} signals in later-stage zebrafish embryos. It also provides an alternative method to generating transgenic zebrafish lines that express Ca^{2+} probes based on fluorescent proteins such as cameleons (Higashima *et al.*, 2003).

Experimental Procedures

Embryo collection

Zebrafish (*Danio rerio*), AB strain, were maintained on a 14 hr light/10 hr dark cycle to stimulate spawning (Westerfield, 1994) and their fertilized eggs were collected as described elsewhere (Webb *et al.*, 1997). Embryos were placed in a custom-designed holding/imaging chamber,

described in detail in Webb *et al.* (1997), in 30% Danieau's solution (17.4 mM NaCl, 0.21 mM KCl, 0.18 mM Ca(NO₃)₂, 0.12 mM MgSO₄·7H₂O, 1.5 mM Hepes, pH 7.2) and maintained at ~28.5°C throughout development.

Preparation of the pSP64TNE-aeq and pSP64TNE-aeq::EGFP plasmids

The apoaequorin plasmid (piP-HE; Inouye *et al.*, 1989) was obtained from Prof. Frederick I. Tsuji (Scripps Institution of Oceanography, University of California San Diego) and the pSP64T-derived plasmid (Kreig and Melton, 1984), pSP64TNE, was obtained from Prof. K.L. Chow (The Hong Kong University of Science and Technology, Hong Kong). All the restriction enzymes were from New England Biolabs Inc. Two plasmids were made; (1) pSP64TNE-aeq, containing the apoaequorin sequence and (2) pSP64TNE-aeq::EGFP, containing a fusion protein of apoaequorin and EGFP (Fig. 1).

For the pSP64TNE-aeq plasmid, PCR was carried out to amplify the apoaequorin sequence (591 bp) from the piP-HE plasmid, using the following oligonucleotide primers:

5'-accagcggccgcatgacaagcaacaataactcagtcagcttacatcagac-3' and 5'-accagaattctaggggacagctccaccgtagag-3', in which two restriction sites, *NotI* and *EcoRI*, were added to the 5' and 3' ends of the apoaequorin sequence, respectively. The apoaequorin sequence was then cloned into the pSP64TNE plasmid using these restriction sites.

For the pSP64TNE-aeq::EGFP plasmid, the apoaequorin sequence was amplified from piP-HE using the

5'-accaggtaccatgacaagcaacaataactcagtcagcttacatcagac-3' and 5'-accaaccggatggggacagctccaccgtagag-3' primers, in which the stop codon was deleted and two restriction sites, *KpnI* and *AgeI*, were added to the 5' and 3' ends, respectively. The apoaequorin sequence was then cloned into the pSP64TNE-EGFP plasmid (the *EGFP* sequence was subcloned from pEGFP-N1; Clontech) using the *KpnI* and *AgeI* restriction sites and fused in-frame at the 5' end of *EGFP*. Further details of the constructs used are available upon request.

In vitro synthesis of aeq-mRNA and aeq::EGFP-mRNA and microinjection

The pSP64TNE-aeq and pSP64TNE-aeq::EGFP plasmids were linearized with *XbaI*. Apoaequorin (*aeq*-mRNA) and apoaequorin-EGFP (*aeq::EGFP*-mRNA) transcripts were made and precipitated using the mMESSAGE mMACHINE[®] sp6 Kit (Ambion) following the protocol recommended by the manufacturer.

Embryos were injected with 400 pg *aeq::EGFP*-mRNA into the blastodisc at the 1-cell stage. The microinjection pipettes and pressure injection system used are described in detail elsewhere (Webb *et al.*, 1997). Bright-field and fluorescence images of embryos at the 256-cell, 12-somite and 20-somite stages, were captured with a Nikon DXM 1200F camera mounted on a Zeiss Axioskop using a Zeiss Plan Neofluar 10x/0.3NA objective. GFP fluorescence was captured using 488 nm excitation/ 507 nm emission.

Testing mRNA toxicity

Different amounts of *aeq*-mRNA (i.e., 500 pg, 700 pg and 1500 pg per embryo) and *aeq::EGFP*-mRNA (i.e., 400 pg, 500 pg, 700 pg and 1500 pg per embryo), were microinjected into the blastodisc of embryos at the 1-cell stage. The mRNA injected embryos were observed at 24 hpf and bright-field images were captured using the Nikon DXM 1200F camera and Zeiss 10x objective described earlier.

Western blot analysis

Embryos that had been injected with 700 pg *aeq*-mRNA or 400 pg *aeq::EGFP*-mRNA at the 1-cell stage were used for Western blot analysis at 2, 4, 8, 12, 18, 24 and 30 hpf. Embryos without mRNA injection were also prepared as a negative control. The chorions and yolks of two embryos per time-point were manually removed using watchmaker's forceps in cold Ringer's buffer (Westerfield, 1994) containing protease inhibitor (1 tablet/ 50 ml; Roche) and then discarded. The remaining embryonic tissues were

then transferred together with cold Ringer's buffer to an equivalent volume of 2X sample buffer (300 mM NaCl, 2% NP-40, 1% Triton X-100, 1% deoxycholic acid, 0.2% SDS, 100 mM Tris-HCl, 2 mM EDTA and 2 mM EGTA). The protein in the samples was denatured at 75-80°C with 5X loading buffer (0.313 M Tris HCl, 50% glycine, 10% SDS, 0.05% bromophenol blue and 10% 2-mercaptoethanol) and then resolved with a 10% SDS-polyacrylamide gel (consisting of a Separating gel: 375 mM Tris base, 0.1% SDS (pH 8.8), 10% acrylamide, 0.15% APS and 0.001% TEMED; and Stacking gel: 125 mM Tris base, 0.1% SDS (pH 6.8), 4% acrylamide, 0.1% APS and 0.002% TEMED). The SDS-PAGE was carried out in running buffer (25 mM Tris-base, 190 mM glycine and 0.1% SDS) at 120 V using a Mini-Protean[®] 3 Cell (Bio-rad). Recombinant *f*-aequorin (20 pg; Shimomura *et al.*, 1989; The Photoprotein Laboratory, Falmouth, MA, USA) was loaded into one lane of the gel as an internal positive control.

The separated proteins were then transferred to a nitrocellulose membrane (Bio-rad) overnight at 4°C in cold transfer buffer (2.4 mM NaOAc, 10 mM MOPS, 20% ethanol, 0.1% SDS) using a Mini Trans-Blot[®] Electrophoretic Transfer Cell (Bio-rad) set at 20 V. For detection of apoaequorin, the nitrocellulose membrane was incubated with blocking buffer (PBS; 136.9 mM NaCl, 2.7 mM KCl, 32 mM Na₂HPO₄ and 8 mM NaH₂PO₄, pH 7.3; containing 0.1% Triton X-100 and 10% goat serum) for 1 h and then probed with a rabbit polyclonal antibody against aequorin (Abcam; at a dilution of 1:2,000 in blocking buffer) for 1 h. Following 4 x 10-min washes in PBS containing 0.1% Triton X-100, the membrane was blocked again, this time for 20 min with blocking buffer, after which it was incubated with a horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz; at a dilution of 1:20,000 in blocking buffer) for 1 h. The membrane was washed for 6 x 10 min with PBS containing 0.1% Triton X-100 and then the presence of apoaequorin protein was visualized with the SuperSignal[®] West Pico Chemiluminescent Substrate (Pierce) and exposed on the Kodak BioMax Light-1 film (Amersham) for ~1 min. Signals on the film were quantified using NIH Image J and normalized using the recombinant aequorin (internal positive control) band, prior to graph plotting.

Preparation of f-coelenterazine

A stock solution of 2 mg/ml *f*-coelenterazine (Molecular Probes) was prepared in methanol. Just prior to use, this stock solution was diluted to either 2 μM in homogenization buffer (20 mM Tris HCl, 10 mM EDTA, 10 mM EGTA, pH 7.5) for the *in vitro* aequorin reconstitution assay, or to 50 μM in 30% Danieau's solution for *in vivo* aequorin reconstitution.

In vitro aequorin reconstitution assay

Embryos (n=10) that had been injected with 700 pg *aeq*-mRNA or 400 pg *aeq::EGFP*-mRNA at the 1-cell stage were manually dechorionated at either 4 hpf or 24 hpf using watchmaker's forceps. The dechorionated embryos were then homogenized for 10-20 sec in 0.5 ml homogenization buffer containing protease inhibitor (1 tablet/50 ml of buffer; Roche), using a sonicator. Extract prepared from uninjected embryos was used as a negative control.

Aequorin luminescence was acquired using an FB15 luminometer (Zylux Corp.). The embryo extract was transferred to a 12 x 75 mm disposable borosilicate glass culture tube and 0.5 ml of 2 μM *f*-coelenterazine was added for the reconstitution of apoaequorin to aequorin. After reconstitution for 1 h, 200 μl of 150 mM CaCl₂ was added to the tube via the automatic injector of the luminometer. The level of luminescence (measured in relative light units, RLU) was then accumulated for 1 min after adding the CaCl₂. The values of total luminescence output were then transferred to Excel 2000 (Microsoft) for data processing and graph plotting.

In vivo reconstitution of aequorin

Embryos were microinjected with 700 pg *aeq*-mRNA into the center of the blastodisc at the 1-cell stage and were then maintained at ~28.5°C until the 64-cell stage. The embryos were then dechorionated manually with

watchmaker's forceps, after which they were transferred to a holding/imaging chamber and then incubated with 50 μ M fcoelenterazine in 30% Danieau's solution. Embryos bathed in this fcoelenterazine solution were then transferred to a custom-built Photon Imaging Microscope System (PIMS; Science Wares, East Falmouth, MA, USA) for data acquisition. An outline of our PIMS and the photon acquisition procedure is described in detail elsewhere (Webb *et al.*, 1997).

Injection of f-aequorin

The protocols used for injecting embryos with aequorin are described in detail elsewhere (Webb *et al.*, 1997). Approximately 4 nl of recombinant f-aequorin (at ~1% in 100 mM KCl, 5 mM MOPS and 50 μ M EDTA) was injected into the top of the embryo's yolk cell at the 128-cell stage. Following microinjection, embryos were transferred to our PIMS for data acquisition.

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