second, we will deliver a very long, very low voltage pulse. The theory is that the first will open pores and the second will not only keep the pores open, but also electro-osmotically drive the aequorin into the cytosol.

As a starting point, we repeated the work of Van Haastert *et al.* (1989), in which a 180 m.w. sugar was electroporated into amoebae of *D. discoideum*. We initially tried using a commercial device, but found that it could not deliver brief enough pulses for our purposes (control of pulse length is in the ms range.) The commercial equipment is intended to deliver long pulses through media several orders of magnitude less conductive than ours, therefore it is built with a very high capacitance and internal impedance. Because we were unable to modify the commercial device, we built our own. It is similar to the commercial one, but ours is able to deliver much faster pulses (*i.e.*, in the μs range as opposed to the ms range) at high voltages. (Fig. 1)

Once the equipment was built, we could begin to search for the electroporation conditions that would result in the greatest percentage of living, electroporated cells. Because aequorin is expensive and somewhat difficult to handle, we decided to begin our search using inexpensive fluorescent dyes, namely lucifer yellow CH (m.w. 487) and FITC-dextrans (m.w. 4400 and 10,000.)

Amoebae of *D. discoideum* are prepared for electroporation according to Van Haastert *et al.* (2). Cell suspensions, electroporation media, and the electroporation chamber are cooled to 0–4°C prior to electroporating. The cell suspension (0.75 ml) is mixed with the dye (0.25 ml) immediately prior to being placed in the electroporation chamber. The "chamber" is created by a 1.0 cm gap between two cylindrical aluminum electrodes, which are encased in plexiglass. The capacitors are charged to the desired voltage and the pulse is delivered through the cell suspension. The geometry of the chamber assures that a uniform field is delivered. After electroporation, the cell suspension is removed from the chamber and mixed with "healing medium" (2) and placed on ice for 10 min. Cells are washed by centrifugation

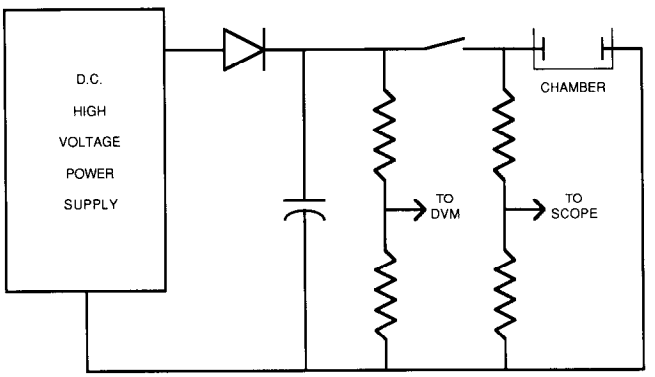

Figure 1. *Schematic of the electroporation device.*

three times in healing medium. The cells are examined for viability and uptake of dye, using both brightfield and fluorescence microscopy. Cell viability and dye uptake are the two criteria for judging the usefulness of each electroporation setting.

Using lucifer yellow, we repeated the results of Van Haastert. Under the reported optimal conditions of two 7 kV pulses for 210 μs, we attained populations in which all living cells are stained, and the number of dead cells is not statistically attributable to electroporation.

Using FITC-dextran 4400, we attained populations in which 25% of the living cells are stained, and the number of dead cells is not statistically attributable to electroporation.

The next step in this research will be to use the larger dye, FITC-dextran 10,000. Once we have attained a high electroporation efficiency with this, we will begin using aequorin.

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A Region of Steady High Calcium at the Vegetal Pole of Medaka Eggs

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Over a decade ago, it was conclusively demonstrated that a remarkable explosive wave of free cytosolic calcium travels from the animal pole to the vegetal pole following fertilization of the egg of the medaka fish (1). Since then it has been shown that such waves traverse a wide variety of, and perhaps all, deuterosome eggs, and may well be the explosive key that blasts the lock off the arrested development of unfertilized eggs. Such waves are propagated by calcium-induced calcium release and traverse a wide variety of eggs at a remarkably similar velocity of around 10 μm/s (2). In spite of the excitement at the confirmation of such a prominent and spectacular phenomenon, it was noted and recorded that the calcium wave lingered for some time at the vegetal pole of medaka. The significance of a domain of high calcium at the vegetal pole in early development, if indeed it was real, was subject to considerable speculation. However, such a phenomenon might be explained by two considerations that bear on the structure and early embryogenesis of the medaka egg. This large (1200 μm diameter) clear egg consists of a central membrane bound yolk mass with a thin (~40 μm thick) peripheral cytoplasm. Thus, the increased signal from the vegetal pole might be explained by the simple fact that there may be more cytoplasm at the vegetal pole or there might be an asymmetric distribution of the calcium recording molecule at this pole, perhaps explained by differential binding to some vegetal cytoskeletal element.

We have continued to use, as in the first report of calcium waves, the chemiluminescent photoprotein aequorin, as our method of choice for recording cytosolic free calcium. This

technique has certain inherent and unique advantages that have been enhanced considerably by recent advances in aequorin chemistry (3). One of these, which was of great significance in addressing the question of the vegetal pole calcium hot spot, was the conjugation of fluorescein to aequorin. This enabled us to do a luminescence to fluorescence ratio correction, settling the question of unequal recorder molecule distribution. We also employed BAPTA type calcium shuttling buffers (4) to disperse any calcium hot spots at the pole.

Table I shows the results of a series of experiments where recombinant aequorin was microinjected into medaka eggs in combination with a fluorescein-labeled aequorin, dibromo-BAPTA, and a KCl blank. The injection technique was Hiramoto's quantitative low-pressure method (5). The procedures used to introduce a micropipette into the thin cytoplasmic layer of the medaka egg were those described by Gilkey (6). Apart from the eggs co-injected with dibromo-BAPTA, all of the others used in this study developed normally and hatched. The aequorin-generated luminescence was observed from eggs in their normal, blastodisc-down orientation with a Zeiss IM-35 inverted microscope, a Nikon Planapo 10/0.45 objective, and a 75 mm optical doublet, which together produced a 6× magnified image on the photocathode of an imaging photon detector or IPD (Imaging Technology Ltd., East Sussex, UK). This device consists of a microchannel plate intensifier with a resistive anode as the positional encoder (7). The raw data from the IPD consists of a sequential record of photon positions and times, measured one at a time. Our analysis involved counting photons in boxes placed at either the vegetal or animal poles, then dividing these numbers by those collected from similar sized boxes placed at the equator of the egg. The IPD was also used to acquire signal from the photoexcitation of fluorescein-labeled aequorin. A similar form of analysis was employed, and the true corrected luminescent ratios between the poles and the equator were calculated.

From Table I it is clear that even after the fluorescence correction, there is approximately three times more luminescence at the vegetal pole than at the equator of the egg. As calcium varies with the cube-root of the luminescence, this equates to a cytosolic free calcium level at the vegetal pole some 50% higher than the background level of the egg. This steady vegetal calcium hot spot persists up to 100 min after fertilization. The introduction of dibromo-BAPTA disperses the calcium in a manner

Table I

Aequorin luminescence ratios (± SEM's) at the animal and vegetal poles of the medaka egg less than 100 min after fertilization

Injectate(s)	n	*Animal pole* Equator	*Vegetal pole* Equator
Aequorin	11	8.7 ± 3.4	3.0 ± 0.7
Aequorin + BAPTA	10	3.6 ± 0.8	1.6 ± 0.3
Aequorin + KCl	4	7.8 ± 1.9	3.0 ± 0.5
Aequorin + Fl-aequorin	9	4.9 ± 1.6	3.4 ± 1.4

Seven × seven pixel wide boxes were positioned at the animal pole or vegetal poles and divided by a similar sized box placed at the equator of the egg. In the case of the aequorin + fl-aequorin co-injection, the luminescence ratios between the poles and the equator were corrected for aequorin distribution.

explained by facilitated diffusion theory (4). This can be clearly seen by the reduction in pole to equator ratios. In addition to the confirmation of the vegetal calcium hot spot, it is also clear that the increase in signal at the animal pole cannot be explained simply by the accumulation of cytoplasm at the pole during embryogenesis. The fluorescent correction indicates that there is also an elevation in animal pole cytosolic free calcium during blastodisc segregation.

We conclude that domains of high free calcium do exist at both the vegetal and animal pole of the medaka egg for at least 100 min after fertilization, and therefore, that calcium gradients exist in the ooplasm of the egg during this time.

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