Labeling of cell membranes and compartments for live cell fluorescence microscopy

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In recent years, the development of new fluorescence and optics technologies has resulted in revolutionary advances in imaging living cells. This chapter describes methods for fluorescently labeling the surface and organellar membranes as well as the spaces they enclose. We describe in detail methods with which we have had direct experience and provide information for other procedures. Fluorescence imaging of cytoskeletal elements are discussed in Chapter 16.

It would be very convenient if one could pull off the shelf a particular fluorescent dye or GFP chimera to label each type of membrane compartment specifically. However, at this time, many useful methods are actually not specific. For example, a method may label several types of compartments or may only "work" for a period of time. In fact, it is sometimes easier to think that one is labeling a "process" than an entity. Therefore, using a method for labeling membrane compartments involves not just a protocol but also some understanding of membrane inter-relationships and dynamics in order to interpret the labeling patterns well.

To begin with, the plasma membrane provides the boundary between the extracellular space and the intracellular space. The intracellular space contains additional membranes which form the boundaries for the organelles. The organelles are relatively small except for the endoplasmic reticulum (ER), which has some similarities to the plasma membrane, in that it is an extensive, continuous membrane. The intracellular space between the plasma membrane and organelles is one continuous space. A special part of the ER, the nuclear envelope, separates one part of this space and forms the boundary of the nucleus while the rest of the intracellular space is the cytosol.

The membranes of the cell undergo complex interactions in which they fuse to combine compartments, bud to create new ones, or move to different locations within cells. For instance, most membrane components are made in the ER, and then pass through the Golgi apparatus to become part of the other organelles, and there is often enormous turnover of plasma membrane through endocytosis and exocytosis. How fusion and budding are regulated, how organelles are able to establish and maintain a functional "identity", how membranes can recover from catastrophic wounds to re-
program themselves (e.g., Terasaki et al., 1997), and what regulates organelle movements are subjects of much current research.

**Labeling of extracellular space / endocytosis / exocytosis**

The extracellular space is easily labeled by including an impermeant fluorescent marker in the sea water. Fluorescent dextrans or calcein work well for this purpose. Dextran are water soluble polysaccharides that can be coupled to various fluorophores and are available in sizes ranging from 3 kDa to >1000 kDa. They are available as lysine-fixable versions which can be fixed in place by formaldehyde. Calcein (Molecular Probes #C-481) is fluorescein modified to have two negative charge groups. This is the smallest fluorescent space marker (MW = 623 Da) with which we have extensive experience. Alexa fluor 350 hydrazide (MW = 349 Da; Molecular Probes #A-10439) has been useful in preliminary experiments.

Extracellular labeling can be used to visualize exocytosis of cortical granules during sea urchin egg fertilization (Terasaki, 1995). The cortical granules are docked at the plasma membrane of unfertilized eggs and are triggered to undergo fusion by the calcium wave at fertilization. Fusion of the large (~1 μm diameter) cortical granules leaves a crater-like depression in the egg surface that can be imaged due to the movement of the extracellular marker in the sea water into the newly created space (Fig. 1A). In some of the original experiments, fluorescently labeled ovalbumin was used as an extracellular marker, but it is probably better to use lower molecular weight dextrans which diffuse into the depression faster. As an example of a labeling protocol, make a stock solution of 10 kDa tetramethylrhodamine dextran at a concentration of 10 mg/ml in sea water (most fluorescent dextrans can be made at this concentration). Store the stock solution in a microfuge tube in the refrigerator; stock solutions appear to be stable for at least several years. As a labeling solution, make a 3:100 dilution to 0.3 mg/ml (~30 μM dye) in sea water. If one is using an injection chamber (see Chapter 10) for imaging, make 1 ml of labeling solution and replace the reservoir of the chamber with the solution. Allow ~5-10 minutes for the solution to equilibrate between the coverslips of the chamber.

During endocytosis, a portion of the plasma membrane buds inward and the lumen of the new intracellular compartment contains fluid that was formerly part of the extracellular space. Uptake of fluorescent markers in the sea water is an excellent
markers for endocytosis. However, there is an important precaution. The smallest endocytic compartments may not contain enough fluorescence to be detectable as an image of a vesicle in light microscopic images, even with good optical conditions.

A prolonged period of endocytotic reuptake of membrane follows the massive exocytosis at fertilization. Extracellular fluorescent markers were used to document the appearance of large endosomes following fertilization, and information about the time course of the endocytosis was obtained by doing pulse chase experiments with dextrans conjugated with different fluorophores (Whalley et al., 1995). Vogel and co-workers have subsequently investigated mechanisms regulating this compensatory endocytosis that occurs after cortical granule exocytosis (e.g. Smith et al., 2000). A quantitative fluid phase assay uses 100 µM 3 kDa tetramethylrhodamine dextran as a fluorescent marker in the sea water (Vogel et al., 1999). Procedures are similar to that described above for imaging cortical granule exocytosis.

Fluorescent extracellular markers were used to assay for membrane rupture in studies of wound healing of the plasma membrane (Terasaki et al., 1997). While observing an egg by confocal microscopy, a large plasma membrane disruption was made in the presence of sea water containing 100 µg/ml fluorescein-conjugated stachyose (a tetrasaccharide; we now prefer calcein because of its lower cost and smaller molecular size). Very little fluorescent marker entered the egg, which is evidence that the disruption was repaired within a few seconds.

**Labeling of plasma membrane / exocytosis / endosomes**

The plasma membrane should be the easiest cell membrane to label because it is the most accessible, but the process is not always completely straightforward. Fluorescent dyes that label the plasma membrane intercalate into the membrane bilayer, and therefore have significant hydrophobicity. Hydrophobic molecules can often pass through membranes and label interior membranes, and are often not soluble in sea water, so that it is more difficult to deliver the dye to the plasma membrane. Effective plasma membrane dyes have electric charges or hydrophilic portions which result in properties that can help circumvent these problems.

Another kind of problem is that labeling of the plasma membrane bilayer will end up labeling intracellular compartments due to continuing endocytosis. This is desired for
some applications, but there are some applications where this is inconvenient or even detrimental. Lastly, microscopic images of the plasma membrane sometimes do not correspond to one’s intuition - when seen in en face views, the microvilli and similar structures along with the undulations into and out of the image plane can make it difficult to interpret confocal images of the plasma membrane.

Currently, the most commonly used dyes for labeling the plasma membrane are probably those of the FM 1-43 family. The pioneering use of these dyes was in investigations of membrane turnover at synapses (Betz and Bewick, 1992). These dyes are soluble in both water and the membrane, but are much less fluorescent in water than in the membrane. They also have the important property that they do not cross membranes. To use them, an aqueous solution of FM 1-43 is put onto cells; after a short while, a dynamic equilibrium is established in which a fraction of the dye has partitioned into and stained the plasma membrane. The dyes can be washed out of the sea water, resulting in loss of plasma membrane staining but retention of dye within any endosomes that have formed while the plasma membrane was stained.

FM 1-43 (Molecular Probes #T-3163) has been used in many studies as an endocytic marker (e.g., Whalley et al., 1995; Wessel et al., 2002; Frejtag et al., 2003). It was also used to visualize exocytosis after fertilization (Terasaki, 1995). As mentioned above, exocytosis of a cortical granule results in the formation of a crater shaped depression in the cell surface. This depression appears as a ring in FM 1-43 labeled eggs for the following reason. The depression lies completely within the optical section of a confocal microscope, which is several microns in thickness. By considering the geometry of the depression, there is more membrane mass (and FM 1-43 molecules) in the wall of the depression (perpendicular to the image plane) than in the floor of the depression (parallel to the image plane); this results in the ring staining (Fig. 1B).

To use FM 1-43 for either endocytosis or cortical granule exocytosis, make a stock solution of 2 mM FM 1-43 in ethanol and store it at -20 °C. Make a 1:1000 dilution of this stock solution in sea water (i.e., 2 µM final concentration) to label the eggs. If using an injection chamber, replace the sea water in the chamber with the FM 1-43 solution and allow 10 min for the labeling to come to equilibrium before adding sperm. Even under conditions of optimal mixing, it takes on the order of 1 min for aqueous FM 1-43 to come
to equilibrium with the plasma membrane; it is important to take this into account in brief, quantitative experiments but is not an issue in long term experiments. FM 1-43 fluorescence is high with fluorescein optics, with some spillover into the rhodamine channel, so FM 1-43 is not an ideal marker for double labeling experiments. There are a number dyes such as FM 4-64 (Molecular Probes, T-3166) or RH 414 (Molecular Probes T-1111) which have similar staining properties but different fluorescent spectra, and could therefore be useful.

Long chain dicarbocyanine dyes such as DiI (DiIC<sub>18(3)</sub>) are insoluble in water but will incorporate into and diffuse in membrane bilayers; these have been widely used in neurobiology to stain the outlines of neurons (Honig and Hume, 1986). They do not cross membranes, and in contrast to FM 1-43, they remain associated once they have become incorporated into a membrane bilayer. The difficulty in using these dyes is delivery. A DiI solution in organic solvent or dried crystals is usually deposited in the region of neurons. This does not seem to work with unfertilized sea urchin eggs because the dye does not get past the vitelline envelope. DiI dissolved in soybean oil can be used to touch the surface of fertilized embryos to label the plasma membrane (D. Burgess, pers. comm.), though this does not seem to work in unfertilized eggs, perhaps due to the vitelline envelope.

A dye similar to DiI, R18 (octadecyl rhodamine Molecular Probes #O-246), has been used to label the sea urchin egg plasma membrane (Smith et al., 2000). R18 is rhodamine with a single 18 carbon alkyl chain attached to it. A stock solution of 10 mM in ethanol was diluted to a concentration of 20 μM in sea water. R18 is insoluble in sea water and forms many small micelles or aggregates that are visible by fluorescence microscopy. Eggs were exposed to this suspension for 5 min then washed to get rid of unincorporated micelles.

R18 was also used for labeling the starfish sperm plasma membrane and observing acrosome formation (Terasaki, 1998) (Fig. 2); this method appeared to work better than with FM 1-43 and offers the possibility of observing sperm egg fusion. To use this method, make a stock solution of 10 mM R18 in ethanol; store at -20 °C. Make a 1 ml of 1:1000 sperm suspension in sea water in a 1.5 ml eppendorf tube, then add 1 μl of R18 stock, invert, and incubate for 20 min. During the incubation time, the R18 micelles /
aggregates apparently collide with and label the sperm. Unlike starfish sperm, sea urchin sperm do not remain active for longer than 5 min after suspension in sea water, and this is not long enough to get good labeling with R18.

There are a large number of related dyes available from Molecular Probes or Avanti Polar Lipids which may have useful properties for plasma membrane labeling. Bodipy sphingomyelin (Molecular Probes #D-7711) is an example. It is soluble in water, and labels the plasma membrane, but in contrast to FM 1-43, does not come out of the membrane when the staining solution is replaced with dye-free sea water. To label eggs, make a stock solution of 1 mM in ethanol, then use 0.1 μM in sea water for 1 min to stain eggs, followed by several sea water washes.

Another approach has been to chemically couple plasma membrane proteins to fluorescent markers. This has been done by removing the vitelline envelope then incubating eggs with Alexa 488 maleimide (Molecular Probes; Smith et al., 2000). Also, it is possible to use Oregon green conjugated concanavalin A, a lectin that binds to specific carbohydrates of glycoproteins on the cell surface (Smith et al., 2000).

In some cases, it would be useful to label just the plasma membrane without labeling of endocytic compartments. This would be most feasible using a membrane protein which is not normally endocytosed. A GFP chimera of casein kinase 1-gamma is localized to the plasma membrane and apparently does not become endocytosed in Drosophila embryos (http://biodev.obs-vlfr.fr/gavdos/); this chimera is untested in sea urchins.

Labeling of the cytosol

The “cytosol” is the intracellular space excluding the nucleus and the organelles, while the “cytoplasm” is the intracellular space excluding the nucleus but including the organelles. The cytosol is easy to label because aqueous fluorescence markers injected into eggs diffuse throughout the cytosol. As with extracellular space labeling, fluorescent dextran and calcein work well.

As an example, to use 10 kDa or 70 kDa fluorescein dextran (Molecular Probes #D-1821), make a stock solution of 10 mg/ml in 100 mM potassium glutamate (or potassium chloride), 10 mM Hepes, pH 7. Make a 2% volume injection (200 μg/ml final concentration); this will result in bright staining. Calcein (Molecular Probes #C-481) is
convenient for some applications because it diffuses rapidly due to its small size (623 Da). To use this, make a stock solution of 0.5 mg/ml in sea water and make a 2% volume injection (i.e., 10 μg/ml final concentration) to obtain bright staining.

Confocal microscope images of fluorescent markers in the cytosol often do not show a uniform distribution. When the cytoplasm is observed with high numerical aperture water or oil immersion lenses (numerical aperture > 1.0), the numerous 1-2 μm diameter yolk platelets, which take up about half of the volume of the cytoplasm, are seen as dark negative images (Fig. 3A; see also next section). Small fluorescent markers (<~25 kDa; Lénárt et al., 2003) injected into the cytosol diffuse passively through the nuclear pores of the nuclear envelope and label the interior of the nucleus, which becomes brighter than the cytosol in confocal microscope images. However, the local concentration within the nuclear space and the cytosol are probably the same; the difference in brightness is due to the cytoplasmic space occupied by the yolk platelets (Terasaki, 1994). Larger markers do not pass through the pores so the nucleus appears dark (Fig. 3B). During mitosis, the yolk platelets are excluded from the mitotic pole regions, so the the mitotic pole regions are brighter than the surrounding regions in confocal microscope images (Terasaki, 2000) (Fig. 3B).

Imaging of the cytosol can be particularly important when using fluorescent ion indicators. With many indicators, binding of an ion causes the amplitude of the excitation and emission curves to change without any change in the shapes of those curves. Calcium green, for instance, has fluorescein-like fluorescence that increases several fold when it binds to calcium; it is coupled to dextran to prevent it from crossing membranes (Molecular Probes #C-3713). When injected into eggs, calcium green dextran diffuses throughout the cytosol; thus, the cytosolic fluorescence increases when cytosolic Ca\(^{2+}\) increases. However, the cytosolic fluorescence also depends on cell thickness or the relative abundance of organelles (which occupy space that would otherwise be cytosol). To correct for this, the calcium dependent fluorescence can be “normalized” or “ratioed” to the amount of cytosol present. This has been accomplished by co-injecting calcium green dextran and tetramethylrhodamine dextran and dividing the calcium green signal by the rhodamine signal at each pixel (e.g. Stricker, 1995; Wilding et al., 1996). This co-injection method is not necessary for other ion indicators such as fura-2 or indo-1; with
these dyes, the shape as well as the amplitude of the excitation or emission curves change, so it is possible to normalize the fluorescence by measuring fluorescence at two different wavelengths.

**Yolk platelets / reserve granules**

Echinoderm eggs have abundant numbers of large organelles of about 1-2 μm diameter that are commonly called yolk platelets or yolk granules. Approximately half the volume of the egg is taken up by these organelles. It has been assumed that these organelles serve as a food supply for the growing embryo, which is the function of similar appearing organelles in embryos of other phyla. However, there is evidence against a nutrient role in sea urchin embryos (Scott et al., 1990) and more recently, evidence for a role in calcium regulation (Churchill et al., 2002), so that these organelles are also called "reserve granules" (Chestkov et al., 1998).

The yolk platelets / reserve granules can be fluorescently stained by nile blue (Danilchik and Gerhart, 1987; Sigma N5632) or nile red (Molecular Probes N-1142) (Fig. 3C). To label by this method, make a stock solution in DMSO (nile blue at 2 mg/ml; nile red at 50 μg/ml; store at room temperature protected from light). Add 1 μl of stock solution to 1 ml of sea water in a glass test tube and swirl. Add sea urchin eggs in ~50 μl and swirl again. The staining should be complete by ~10-20 min. The dye can be left in the sea water, and the fluorescence of either dye is observed with rhodamine filters. The time required for uniform staining is ~10 min. To stain eggs that are already loaded in a microinjection chamber, simply change the large reservoir to sea water with a 1:1000 dilution of nile blue / nile red stock solution. The time for complete staining is somewhat longer (~20 min) due to restricted access. These dyes do not label yolk platelets in starfish oocytes, where they only stain sparse large vesicles in the cortex (these do not undergo exocytosis at fertilization).

**Mitochondria**

In many cell types, mitochondria are easily labeled by water soluble, lipophilic, positively charged fluorescent molecules which cross the plasma membrane and become concentrated in mitochondria due to the large negative membrane potential of the mitochondria. The first of these dyes to be described was rhodamine 123 (Johnson et al.,
1980), but many other dyes have these properties, including short chain dicarbocyanine dyes and the “mitotracker” series of dyes.

Mitochondria have been labeled in ascidian eggs using DiOC₃(3) (Molecular Probes, #D-14730) or Mitotracker Green FM (Molecular Probes, #M-7514) (e.g., Roegiers et al., 1999). Eggs are incubated for 15-20 minutes (0.5 µg/ml DiOC₃(3) or 1 µM mitotracker) in sea water buffered with TAPS, pH 8.3. Both of these dyes have fluorescein like fluorescence, but mitotracker is also available with different excitation / emission spectra. Mitotracker Green FM has been used to stain mitochondria in sea urchin embryos using similar conditions (200 nM for 10 min at 15 °C in the dark; Coffman and Davidson, 2001).

**Other organelles**

It would be convenient to have fluorescent markers for some other organelles found in echinoderm eggs. We are unaware of any method to specifically label the cortical granules in live eggs. They can be visualized in negative image by injecting cytosolic markers (e.g., Terasaki, 1995). There are large "acidic vesicles" in the cytoplasm of eggs. These are pigmented in *Arbacia punctulata* eggs and can be distinguished by transmitted light microscopy. The dye nile blue which labels yolk platelets in sea urchin eggs, does not label yolk platelets in starfish oocytes. Instead, it labels large vesicles in the cortex which are not exocytosed at fertilization. We are unaware of methods to label lysosomes except perhaps by a specific GFP chimera targeted to the lysosome.

**Endoplasmic reticulum**

The endoplasmic reticulum (ER) is a membrane compartment concerned with synthesis of membrane proteins, secretory proteins, and membrane lipids, as well as with regulation of intracellular calcium. As mentioned above, it is distributed throughout the cell and includes the nuclear envelope.

Short chain dicarbocyanine dyes such as DiOC₆(3) are effective for staining ER in cultured fibroblasts (Terasaki et al., 1984). These dyes stain many organelles nonspecifically and the ER pattern is possible to identify in the thin spread regions of fibroblasts, but it is not possible to do this in spherical echinoderm eggs. Instead, the ER in echinoderm eggs has been successfully stained by two other methods: injecting an oil drop saturated with a long chain dicarbocyanine dye, or expressing a GFP chimera.
**Dil method** The name “Dil” refers to either DiIC<sub>18(3)</sub> or DiIC<sub>18(3)</sub> (D-384, D382; Molecular Probes). Both are dicarboxycyanine dyes that consist of a fluorescent portion and two long alkyl chains, and both can be used to label the ER. Dil incorporates into the membrane bilayer through intercalation of the long hydrocarbon chains (Axelrod, 1979). Once it is incorporated in the bilayer, the dye is able to diffuse freely within the bilayer, but does not transfer out of the membrane. Dil was first used to trace the plasma membrane of neurons by Honig and Hume (1986). Since then it and several other related dyes have been used extensively for this purpose (see Haugland, 2002, for references).

For labeling the ER, Dil is dissolved Wesson soybean oil and microinjected into eggs. The oil droplet contacts and stains many different membranes at the site of contact. However, of the organelles in the cell, the only organelle that has extensive continuity is the ER. The dye spreads in the continuous membranes of the ER, so that in regions away from the oil drop only the ER is stained.

One limitation of this method is that bright punctate labeling accumulates with time and represents dye leaving the ER network via membrane traffic with the Golgi apparatus; this first becomes noticeable ~1 hr after injection. Another possible disadvantage is that the oil drop usually must be relatively large to get bright staining. In our experiments, the oil drop was ~10% of the size of the egg, and this sometimes was a barrier for imaging, or caused the mitotic apparatus to become displaced. If necessary, the oil drop can be removed from the egg by suction with a micropipet, after the Dil has diffused into the ER.

Using Dil in sea urchin eggs, we observed a transient change in the ER structure at fertilization in which large cisternae became more finely divided (Terasaki and Jaffe, 1991). We also fixed eggs in glutaraldehyde and then injected Dil-saturated oil drops. The fixation eliminates spread of the dye by membrane traffic. We observed that the dye spread throughout the fixed eggs, providing stronger evidence that the internal cisternae, cortical network, and nuclear envelope are all part of one membrane (Jaffe and Terasaki, 1993). We also observed that the dye spread throughout eggs fixed 10 min after fertilization, but that it did not spread significantly from the oil drop in eggs fixed 1 min after fertilization. This provided evidence that the ER is fragmented at the time of calcium release during fertilization, and that the ER subsequently regains its continuity.
This was corroborated by later experiments with GFP-KDEL described below. The DiI method has also been used in ascidians (Speksnijder et al., 1993) and starfish (Jaffe and Terasaki, 1994) to label the ER and observe its dynamics during maturation, fertilization and early development.

To prepare a saturated solution of DiI in oil, crystals of DiI (several milligrams) were sprinkled into the bottom of a 1.5 ml microfuge tube. Approximately 300 µl of Wesson oil (purchased from a grocery store) was then added and the tube was inverted a few times. DiI dissolves relatively slowly, so it should be left for a few hrs or overnight, by which time the the oil should be a bright red solution, with some crystals remaining in the bottom of the tube. To remove the crystals and transfer the saturated oil to a new tube, centrifuge at 10,000 g for a few minutes. The DiI in oil is then stored at room temperature protected from light. This is good for at least several months. Because Wesson oil eventually becomes rancid, it is probably better to use a bottle that has been purchased within the last 6 months to 1 year.

In the original experiments, we used Wesson soybean oil. At the time, this was unavailable in Europe, so an attempt was made with corn oil; DiI dissolved better in the corn oil, but when this was injected, most of it remained in the oil drop and did not spread into the ER (A. Speksnijder, unpublished observations). We then tested different oils but most appeared to work as well as soybean oil in sea urchin eggs (e.g., olive, canola, walnut).

DiI fluorescence is best observed using rhodamine fluorescence filters. For some double labeling experiments, it would be useful to observe the ER with fluorescein fluorescence filters. This can be done by using the long chain dicarbocyanine “DiO” (DiOC18(3); Molecular Probes #D275). This dye does not dissolve directly in oil as does DiI, so the procedure is to dissolve it first in DMSO at 10 mM, then to make a 3% dilution of this solution into Wesson oil (Feng et al., 1994).

**GFP method** The green fluorescent protein (GFP) from the jellyfish *Aequorea* has revolutionized cell biology and is described in detail in other chapters. The construct "GFP-KDEL" improved the specificity of labeling of the ER over the DiI method (Terasaki et al., 1996). GFP-KDEL, as with most membrane proteins or secretory proteins, cannot be injected as a recombinant protein and must be synthesized by the
cell's own machinery. Currently, the best way to accomplish this in echinoderms is to inject mRNA encoding for the protein. We provide a detailed discussion of what was involved in designing and expressing GFP-KDEL as a guide for designing and expressing other GFP chimeras of membrane proteins or secreted proteins.

The GFP-KDEL labeling method was based on how proteins are thought to be targeted to, and retained in, the ER. As a protein is being synthesized in the cytoplasm, a "signal sequence" protrudes from the ribosome and is recognized by the signal recognition particle (SRP). The SRP stops translation until the SRP / ribosome complex binds to a receptor on the ER, where translation resumes and the nascent protein is directed into the ER. GFP is a soluble protein of the cytosol, and was targeted to the ER as a soluble protein of the ER lumen. We used an N terminal signal sequence from ECas/PD1, a luminal ER protein of sea urchin eggs (Lucero et al., 1994). It was necessary to make a second alteration in order for GFP to be retained in the ER. It is thought that the default fate for soluble proteins in the ER is to be processed by the Golgi and secreted out of the cell. Many resident ER luminal proteins have a characteristic four amino acid C terminal retention sequence KDEL (lysine glutamate aspartate leucine), and artificial proteins with this C terminal sequence are retained in the ER (Munro and Pelham, 1987). To ensure that our GFP construct remained in the ER, we designed it with a C terminal KDEL retention sequence.

Injection of mRNA is a routine procedure in Xenopus oocytes (Wormington, 1991), and many of the methods and reagents that have been worked out for Xenopus can be applied to echinoderm eggs. We chose the vector pSP64-RI (Tang et al., 1995), variants of which are widely used for Xenopus oocytes. This vector has a promoter for the RNA polymerase SP6 for making mRNA in vitro (Krieg and Melton, 1984). We were initially interested to observe the ER during fertilization. Expression of proteins for fertilization experiments can be easily accomplished with starfish oocytes by injecting mRNA into immature oocytes, incubating overnight to allow for expression, maturing the oocyte, then fertilizing (Shilling et al., 1990). This is not as easy in sea urchins, due to low rates of protein synthesis in unfertilized sea urchin eggs, but incubation for >6 hrs has resulted in enough GFP chimera expression for some fertilization experiments (J. Ellenberg, pers. communication).
The GFP-KDEL construct was made by John Hammer (NHLBI, NIH) starting with DNA coding for the signal sequence of ECast/PDI (provided by H. Lucero and B. Kaminer) and DNA coding for the S65T mutant of GFP (provided by R. Tsien). This GFP mutant is brighter and folds faster after its synthesis (Heim et al., 1995), so it is clearly desirable to use this mutant for GFP chimeras (the EGFP variant (Clontech) is preferable for mammalian cell studies because it folds better at 37 °C than wild type or S65T GFP but has no advantage for echinoderms).

Oligonucleotide sequences were designed so that PCR reactions would add two desired sequences as well as provide restriction sites that would allow the PCR products to anneal to each other, and also to integrate into the multiple cloning site of the vector. The two sequences added by this procedure were the C terminal KDEL sequence and the "Kozak sequence" from sea urchin ECast/PDI. The Kozak sequence is the first few nucleotides preceding the start codon (ATG) and the first nucleotide after the start codon (Kozak, 1999); these nucleotides are thought to be involved in ribosome association and have been found to have a very significant effect on expression of exogenous proteins. If a construct from another species does not express well, we, in agreement with others, have found that changing the Kozak sequence (the pre-ATG nucleotides seems to be sufficient) to one of the recipient species will frequently improve the expression level.

The plasmid containing GFP-KDEL was grown in bacteria and handled by standard techniques to isolate and cut DNA for making mRNA. We have used the mRNA synthesis kit from Ambion (mMessage mMACHINE; Austin, TX), which makes 10-20 µg mRNA from 1 µg DNA. The mRNA is resuspended in nuclease free water supplied in the kit, and then stored in 2 µl aliquots at -70 °C. RNA is easily degraded by RNases in the environment, so microfuge tubes and pipetman tips from boxes designated "for RNA use only" were used, and gloves were used to handle the tubes. The mRNA aliquots can be frozen and thawed several times.

Experimental results from Terasaki et al. (1996) are summarized here. We found that the oocytes tolerated an injection of ~10-20 µg/ml final concentration of GFP-KDEL mRNA; higher concentrations led to cell death within a few hours. We monitored the appearance of GFP fluorescence and found a lag of 1-2 hrs, then a linear increase phase, and a plateau by about 24 hrs. The lag is probably due to the time needed for recovery
from injection, synthesis and folding. The fluorescence of GFP-KDEL expressing eggs was compared with that of eggs injected with a known amount of fluorescein dextran. By using published values for the quantum yield and extinction coefficient of fluorescein and GFP, the plateau concentration of GFP in the cell was estimated as 0.7 μM; the concentration in the ER lumen must be higher because the ER occupies a small fraction of the total cell volume. When imaged at high magnification, the GFP-KDEL fluorescence pattern was the same as seen previously with DiI (Jaffe and Terasaki, 1994), but there was no apparent transfer to other compartments with time such as seen with DiI.

For studies of fertilization or development, it is necessary for the oocytes to undergo "maturation". Starfish oocytes are arrested at prophase of meiosis I ("GV stage"); we injected the mRNA at this stage. Starfish oocytes are induced to mature by the hormone 1-methyladenine and becomes competent to undergo normal fertilization at ~30 min (at the time of first metaphase). The eggs remain at first metaphase for another ~30-60 min (depending on the temperature). Fertilization should be carried during this period prior to first polar body formation.

During starfish maturation and fertilization, changes in GFP-KDEL labeled ER organization were identical to those seen earlier with DiI (Jaffe and Terasaki, 1994). In immature oocytes, randomly oriented cisternae were present, while in mature oocytes, circular profiles were common, which are likely to correspond to incomplete spherical shells around yolk platelets. After fertilization, there was a transient disruption of this pattern, lasting about 10-15 min. As with DiI, the resolving power of the confocal microscope was not quite sufficient to determine whether the ER had become converted to tubules or had become vesiculated. We used FRAP (fluorescence redistribution after photobleaching) to address this issue.

In FRAP experiments, the fluorescence in a small region of the cell is photobleached by exposure to high intensity excitation light. The redistribution of fluorescence from the unbleached regions is then monitored. The way in which the redistribution occurs can give very useful information, for instance on the kinetics that underlie steady state distributions. GFP is very well suited for photobleaching experiments; this is probably because the fluorophore of GFP is buried within the protein (Yang et al., 1996; Ormo et
al., 1996), so that photodynamic damage involves the GFP protein rather than neighboring proteins.

When a small region of GFP-KDEL was photobleached in unfertilized eggs, the fluorescence in that region recovered within about 1 min. A similar recovery from photobleaching was seen in eggs 20 min after fertilization. However, when eggs were photobleached 1 min after fertilization (when the structural change could already be detected), the fluorescence took approximately 10 min to recover. These results are consistent with a continuous network of ER that normally provides pathways for diffusion throughout the cell, but which is transiently disrupted at the time of fertilization. The disruption is very probably related to the release of Ca\textsuperscript{2+} from the ER which occurs at fertilization.

In later experiments, mRNA for GFP-KDEL was injected into sea urchin eggs. Due to the increase in protein synthesis that occurs after fertilization, GFP fluorescence developed so that it became possible to observe labeling after about the 4th or 5th cleavage (Terasaki, 2000). It was found that the ER did not vesiculate during mitosis, and that it accumulated around the spindle poles (Fig. 4A).

**Golgi apparatus**

Proteins and lipids synthesized in the ER must pass through the Golgi apparatus before they become part of other organelles. By electron microscopy, there are many stacks of Golgi apparatus scattered throughout the cytoplasm of echinoderm eggs; later in embryonic development, when the cells take on epithelial characteristics, there appears to be only one large Golgi complex (Gibbins et al., 1969).

Bodipy ceramide (Molecular Probes #D-3521) is a fluorescent marker for the Golgi apparatus that can be applied from the outside of cultured mammalian cells (Pagano et al., 1991); however, it does not appear to work well in echinoderm eggs. The most effective way to label the Golgi apparatus has been with GFP chimeras (Terasaki, 2000). The chimeras were originally used in cultured mammalian cells and were targeted to the Golgi apparatus using a galactosyl transferase fragment or an ELP mutant (KDEL\textsubscript{m}) which is retained in the Golgi (Cole et al., 1996). mRNA was injected into unfertilized sea urchin eggs which were then fertilized. Staining was bright enough for imaging by around the 4th cleavage. In time lapse sequences, the scattered Golgi stacks underwent
large changes during mitosis, where most of the staining appeared to relocate to the ER, and the Golgi stacks re-formed after the end of mitosis (Fig. 4B). Time lapse observations also showed that the scattered Golgi coalesced into one Golgi after the 9th cleavage, which is one cleavage cycle before the embryo secretes a hatching enzyme.

Nucleus

The nucleus is bounded by the nuclear envelope, a part of the endoplasmic reticulum that has closed in on itself. The membranes of the nuclear envelope form a permeability barrier between the nucleus and the cytosol. Passage across the nuclear envelope occurs through the nuclear pores; small molecules of less than ~25 kD can diffuse passively through the pores (Lénárt et al., 2003), while larger molecules must be actively transported. When large fluorescent dextrans are injected into the cytoplasm, the nucleus remains dark (e.g. Fig. 3B). Smaller fluorescent dextrans enter the nucleus, and reach a level that is approximately twice as bright as the surrounding cytoplasm. This is due to the absence of organelles in the nucleus and to the presence of the large abundant yolk platelets in egg cytoplasm (Terasaki, 1994).

The nuclear envelope is easily visible by transmitted light microscopy, but it has been useful to label its components (nuclear pores, lamina) by GFP chimeras to investigate how the nuclear envelope breaks down during meiosis or mitosis (Terasaki et al., 2001; Lénárt et al., 2003).

During meiosis and mitosis, the cellular DNA becomes condensed into the chromosomes so that the DNA can be efficiently partitioned between two daughter cells. The most convenient way to label chromosomes remains incubation in sea water with either Hoechst 33258, 33342 or DAPI (H-33258, H33342, D-9564 from Sigma Chemical Co., St Louis or H-1398, H-1399, D-1306 from Molecular Probes). See Chapter 16 for details. The main disadvantage of the Hoechst dyes or DAPI is the requirement for ultraviolet excitation, which is not available on many confocal microscopes. Other methods for labeling chromosomes require microinjection. Fluorescently labeled chromosomal proteins such as rhodamine histone (Minden et al., 1989), or GFP chimeras can potentially be used.

YOYO-1 (Molecular Probes #Y-3601) binds directly to nucleic acids and can be used to label chromosomes, but there is a large background staining of RNA in the cytoplasm.
YOYO comes as a 1 mM solution in DMSO. Make a 1:10 dilution (to 100 μM) in an injection buffer consisting of 100 mM potassium glutamate, 10 mM Hepes, pH 7. Make a 3% volume injection, resulting in a final concentration in the egg of 3 μM. There are a number of dyes related to YOYO-1 that are available from Molecular Probes and which may also be useful.

Another possibility is Oregon Green dUTP (Molecular Probes #C-7630), a fluorescent nucleotide that is incorporated into newly synthesized DNA (Carroll et al., 1999). This produces bright staining but only after one round of cellular DNA synthesis (Fig. 5). Oregon Green dUTP comes as a 1 mM solution in 10 mM Tris, 1 mM EDTA, pH 7.6. Make a 100 μM stock solution in the injection buffer described above and then make a 1% volume injection to a final concentration in the egg of 1 μM. Observe the fluorescence with fluorescein optics.

**Microscopy considerations**

The way in which the egg or embryo is kept on the microscope stage is critical for the success of a live cell experiment. The observation chamber must be conducive for normal development as well as for microscopy. We most frequently use the same chamber used for microinjection (see Chapter 10) though there are certainly other ways. The eggs are held securely between two coverslips and can be observed by oil or water immersion objective lenses. The eggs can be injected, fertilized and their development observed in the same chamber. There does not seem to be much difference in using an upright versus inverted microscope, though in cases where it is desired to image while injecting, it is much easier to use an upright microscope configuration.

Another very significant problem in live cell imaging is photo-toxicity. From its excited state, the fluorescent molecule sometimes does not decay to make light but instead reacts with molecular oxygen to form triplet state oxygen which can then react with and damage neighboring molecules (e.g., Tsien and Waggoner, 1994). In live cell imaging experiments, the challenge is to obtain useful data without perturbing the system by photo-toxicity. It is important to use the microscope efficiently so as to use minimal amounts of light in obtaining data (e.g., Terasaki and Dailey, 1994). It is also important to be able to evaluate whether photo-toxicity is affecting the process or structure that one is studying. With regards to this problem, there is an advantage to working with
echinoderm eggs or embryos. It is possible to fertilize and watch normal development over relatively short periods which provides a straightforward and easy way to evaluate whether damage has occurred. As an example, if one were imaging events in fertilization, one way to test for photo-toxicity is to let the embryo develop and see if the first cleavage occurs on time and in a normal fashion.

A related issue is that the labeling procedure itself can affect normal processes. GFP chimeras of endogenous proteins are of necessity over-expressed and could cause toxicity. Fluorescent dyes can be used at higher concentrations to provide brighter signals but may be toxic at the high levels. These should also be easier to evaluate in echinoderm embryos by observing whether the labeling procedures are compatible with normal development.

Future Directions

There will most certainly be continuing improvements in techniques for live cell imaging. The most likely advances will involve fluorescent proteins, from the development of useful GFP chimeras to new types of fluorescent proteins. It also seems likely that there will be an increasing use of light microscopy to make quantitative physiological measurements, such as with sensors for kinases, etc. With multiphoton microscopy, imaging of tissues and older embryos should become more feasible, and fluorescent labels for components of the extracellular matrix, and specialized structures and tissues such as the skeleton or nervous system should become available. Optical methods for sperm should become improved as well. Lastly, mundane details such as keeping embryos alive or immobilized on the microscope can enable the newest, most exciting technique to be used well!
Acknowledgements
We thank several colleagues for telling us about fluorescent probes: Steve Vogel for octadecyl rhodamine, Kristien Zaal for bodipy sphingomyelin, and John Newport for Oregon green dUTP.

References
surface area is not required for cell division in early sea urchin development. *Dev. Biol.* 259: 62-70.


Figure Legends

Figure 1. Simultaneous visualization of cortical granule exocytosis by A) an extracellular space marker and B) a plasma membrane marker. A sea urchin egg was fertilized in the presence of 0.2 mg/ml Texas Red conjugated ovalbumin (which behaves similarly to fluorescent dextrans) and 2 µM FM 1-43, both in the sea water. A 63x 1.4 NA objective lens of a confocal microscope was focused on the surface of the egg adjacent to the coverslip; the fluorescence of the ovalbumin and FM 1-43 was imaged simultaneously through use of a double labeling filter set. As described in the main text, cortical granule exocytosis results in a crater-like depression in the cell surface. Labeling of this depression by an extracellular marker results in a disk pattern while labeling by a plasma membrane marker results in a ring pattern. Bar = 10 µm. Modified from Terasaki, 1995.

Figure 2. Visualization of the sperm acrosomal process by labeling of the plasma membrane. Starfish sperm were mixed with R18 (octadecyl rhodamine), and after an incubation period, were added to eggs previously injected with calcium green dextran and imaged by confocal microscopy (20x, 0.5 NA objective lens). The jelly layer surrounding the egg induces the sperm acrosome reaction, resulting in extension of the acrosomal process, which in starfish is particularly long (~10 µm). In the left panel, the acrosomal processes of two sperm have extended to contact the egg surface (arrowheads); the right panel shows the outline of the calcium green injected egg.

Figure 3. A) Labeling of the sea urchin egg cytosol by injection of fluorescein conjugated 70 kD dextran. The fluorescence was observed by confocal microscopy at high zoom with a 63x 1.4 NA objective lens. The yolk platelets are abundant organelles and because of their relatively large size (1-2 µm diameter), the space each yolk platelet occupies is clearly seen as a dark oval region in the cytosolic fluorescence image (one of the yolk platelets is indicated by the arrowhead). B) Labeling of the cytosol before and during mitosis. An egg injected with fluorescent 70 kD dextran was fertilized and observed after a few cleavage cycles using imaging conditions as in the previous panel but at a lower zoom. The large dextran does not cross the nuclear pores, so that the interior of the nucleus appears dark (left panel); this image was taken a few minutes before nuclear envelope breakdown, and the two spindle pole regions have become
brighter due to exclusion of yolk platelets from these regions (arrowheads). After the nucleus has broken down (right panel), the fluorescent 70 kDa dextran enters the former nuclear region. This region becomes brighter than the peripheral cytoplasm due to the absence of yolk platelets. C) Labeling of yolk platelets by nile blue observed by confocal microscopy with a 63x, 1.4 NA objective lens.

**Figure 4.** A) Labeling of the ER by GFP-KDEL. Sea urchin eggs were injected with mRNA coding for GFP-KDEL then fertilized. Images were obtained by confocal microscopy at low zoom with a 63x, 1.4 NA objective lens as the blastomeres went through the sixth cleavage; the timing for images is indicated (min:sec). The ER undergoes a reversible accumulation at the spindle poles during mitosis; the breakdown and reformation of the nucleus can also be seen. Bar = 10 µm. B) Labeling of the Golgi apparatus by GFP-KDEL Rm. Eggs were injected with mRNA for GFP-KDEL Rm then fertilized. Fluorescence was observed as in the previous panel while the embryos underwent seventh cleavage; the timing of the images is indicated. During interphase, the Golgi is present as numerous, separated stacks. The number and size of these stacks decrease drastically during mitosis; the stacks then return as the blastomeres exit from mitosis. Bar = 10 µm. Modified from Terasaki, 2000.

**Figure 5.** Labeling of chromosomes by a fluorescent nucleotide. A sea urchin egg was injected with Oregon green dUTP then fertilized. The fluorescent nucleotide becomes incorporated into the DNA during the S period of the cell cycle. The embryo was imaged after several cleavage cycles by confocal microscopy with a 63x, 1.4 NA lens. In this image, a nucleus with condensing chromosomes is seen at the top, a cell in early metaphase is at left top, a cell in metaphase is seen at right middle, and an anaphase pair is seen at the left bottom.
Table 1. Some representative dyes for labeling membrane compartments. Other useful dyes are described in the text. IB = intracellular buffer (100 mM potassium glutamate, 10 mM Hepes, pH 7).

<table>
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<th>Dye</th>
<th>Excit max</th>
<th>Emiss max</th>
<th>Stock solution</th>
<th>Solvent</th>
<th>Application</th>
</tr>
</thead>
<tbody>
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<td>Fluorescein dextran</td>
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<td>sea water IB</td>
<td>extracellular intracellular</td>
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<td>517</td>
<td>0.5 mM</td>
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<td>extracellular intracellular</td>
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<td>ethanol</td>
<td>plasma membrane</td>
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<tr>
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<td>578</td>
<td>10 mM</td>
<td>ethanol</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>Dil</td>
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<td>565</td>
<td>2.5 mg/ml saturated</td>
<td>ethanol Wesson oil</td>
<td>plasma membrane ER</td>
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<tr>
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<td>516</td>
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<td>636</td>
<td>50 μg/ml</td>
<td>DMSO</td>
<td>yolk</td>
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</table>
Figure 1
Figure 2
Figure 4A
Figure 4B
Figure 5