Confocal Microscopy of Intracellular Calcium Dynamics during Fertilization

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During fertilization in all animals investigated, fully developed eggs or meiotically immature oocytes must generate a proper change in their intracellular free calcium concentrations ($[Ca^{2+}]_i$) to develop normally (31). The first images of a fertilization-induced calcium response were obtained in fish oocytes that had been microinjected with the calcium-sensitive photoprotein, aequorin (4). Since then, aequorin has been used to monitor calcium dynamics in several other species (13). However, with the introduction of confocal laser scanning microscopy (CLSM) and a wide collection of calcium-binding fluorescent probes, there has been a rapid growth in the number of investigations that use CLSM to track changes in $[Ca^{2+}]_i$ during fertilization (24).

This column specifically addresses changes in $[Ca^{2+}]_i$ that occur within fertilized eggs imaged by commonly available confocal laser scanning microscopes (i.e., those employing single-photon excitations provided by a low-power laser). It serves as an adjunct to more broadly based reviews covering confocal microscopy and calcium dynamics in eukaryotic cells subjected to diverse stimuli (15,27). Several advantages and potential drawbacks to consider when conducting CLSM studies of fertilization-induced calcium dynamics are discussed. In addition, a few of the major findings of such investigations are described, and some possible future directions for CLSM analyses are explored.

Why Use Confocal Microscopy and Calcium-Sensitive Fluorescent Probes?

One advantage of confocal microscopy is its improved resolving power compared to that afforded by conventional epifluorescence microscopy. Based on various calculations, CLSM provides approximately a 40% enhancement in lateral resolution (i.e., "x-y" resolution within the optical plane of focus) so that specimen components occurring somewhat less than 200 nm apart are capable of being resolved as separate (12). In fact, various ancillary techniques can be used to provide enhanced resolving capabilities that might even allow resolution down to 50 nm in some test specimens imaged by CLSM (16). However, when applied to relatively thick biological samples such as eggs, confocal microscopy may fail to achieve the marked enhancements in lateral resolution that are observed in optimized test specimens. This is because microscope objectives with relatively low numerical apertures are often used with such specimens to obtain adequate working distances for viewing internally localized structures.

The confocal microscope also affords a marked improvement in vertical resolution (i.e., along the z-axis of the speci-

men) over that achieved using conventional microscopic methods. Thus, optical sections of approximately 0.5 to several micrometers thickness can be obtained in confocal images of eggs. This in turn allows internal regions of eggs to be imaged by CLSM in a way that is not readily matched by conventional epifluorescence microscopy.

As for confocal-compatible fluorophores, dual-wavelength calcium indicators such as fura-2 and indo-1 have been successfully used to track calcium dynamics in CLSM systems possessing modified optics and UV lasers (9,14). Similarly, the so-calledameleon probes, which use fluorescence resonance energy transfer between mutants of green fluorescent proteins fused to calmodulin and calmodulin-binding peptide, have been genetically engineered for imaging $[Ca^{2+}]_i$ in a confocal microscope equipped with two-photon excitation (3).

However, the most commonly used fluorophores are single-wavelength indicators such as fluo-3, Calcium GreenTM, FURA RedTM, and Oregon Green[®] BAPTA (8). Such probes are commercially available and well suited for imaging analyses that do not require modifications of the CLSM system. Additional advantages of these calcium-sensitive fluorophores include a selection of various calcium-binding affinities that in turn can facilitate the tracking of calcium transients over a range of amplitudes. In addition, some indicator dyes (e.g., Calcium Green and Oregon Green BAPTA) come conjugated to high molecular weight dextrans that help to minimize the loss of dye reactivity via compartmentalization processes (27).

Although such properties are indeed beneficial, perhaps the most attractive attribute of fluorescent calcium reporters is the addition of acetoxymethyl (AM) esters that make the dyes membrane permeable (8). When using such AM forms of the calcium indicator, various cell types can be simply soaked in media containing the probe before CLSM analyses.

What Are the Limitations and Potential Artifacts of CLSM Analyses of Calcium Dynamics?

Confocal analyses of calcium dynamics can be greatly constrained by the speed of image acquisition because full-frame images may take as long as several seconds to collect in conventional CLSM. To increase temporal resolution, restricted regions of the field of view can be captured at progressively higher frequencies, and when using a single line-scan mode, acquisition rates of only a few milliseconds can be accomplished. Alternatively, some more advanced forms of confocal microscopes are designed to acquire full-frame images at video rates of 30 images per second or faster (29).

Additional limitations of confocal analyses include the fact that fluorescent indicators must bind the ion they are designed to monitor. In doing so, such probes may significantly alter intracellular calcium dynamics or even eliminate calcium transients owing to the buffering effects of the dyes (7). However, if the fluorophores are kept at low enough concentrations, it is possible to use CLSM to monitor intracellular calcium levels and still maintain normal physiological responses and cellular functioning (21).

Calcium-sensitive fluorophores are also subject to compartmentalization events within the dye-loaded cell that limit the useful lifetime of these indicators during long-term time-

lapse imaging analyses (5). Although compartmentalization cannot be fully eliminated, dextran-conjugated forms of calcium-sensitive dyes are available in a variety of molecular weights, and such probes have been successfully used to track calcium levels for several hours (21).

Aside from potential problems related to calcium buffering and compartmentalization, the single-wavelength fluorophores commonly used in CLSM studies can be greatly affected by “dye-loading and differential pathlength” artifacts, where differences in fluorophore fluorescence may not necessarily reflect actual differences in free calcium levels (27). To compensate for such artifacts, it is possible to conduct ratio-metric analyses with dual-wavelength indicators such as fura-2 or indo-1, if the CLSM system is capable of providing UV excitation.

In the absence of a UV confocal system, the fluorescence of single-wavelength indicators can be normalized to reduce the effects of dye-loading/pathlength artifacts. For example, each image from the time-lapse run can be divided by the initial frame to provide a set of single-channel “ratioed” images that display relative changes in fluorescence over time (Figure 1A). However, such methods require that the loaded cell remains stationary and does not undergo changes in pathlength or dye distribution during the time-lapse run. As a modification to this single-channel method, two single-wavelength calcium indicators—for example, fluo-3 and Fura Red—can be used to track calcium dynamics even in nonstationary cells that display pathlength changes during imaging (11). Alternatively, a calcium indicator such as Calcium Green can also be co-loaded with a non-calcium-sensitive fluorophore to collect pairs of images that are subsequently ratioed by the dual-channel method to correct for dye loading/pathlength artifacts in a wide variety of specimens (Figure 1, B and C).

What Have CLSM Analyses of Fertilization-Induced Calcium Dynamics Revealed?

Confocal images of sea urchin eggs undergoing fertilization were the first to demonstrate that the rise in intracellular free calcium spreads throughout the ooplasm rather than being restricted to just the periphery (26). In sea urchins and many other animals investigated (24), the calcium transient elicited by fertilization typically propagates as a point-source wave, although a few exceptions to the wave-like transmission pattern have been noted (18,20). Following calibration of the fluorescence signals (10), the amplitudes of such fertilization responses usually peak at several hundred nanomolar to a few micromolar $[Ca^{2+}]_i$, depending on the species examined (24).

Confocal data sets depicting fertilization-induced calcium waves can be simply shown graphically to indicate changes in $[Ca^{2+}]_i$ over time. Alternatively, to view spatial aspects of the calcium response, time-lapse confocal runs can be assembled as 2-D montages (Figure 1, A–C and E), and such images provide valuable information regarding propagation mechanisms based on precise analyses of wavefront shapes (30).

As a supplement to the 2-D montages, each time-lapse confocal run can be viewed as a “cleaved 3-D rendering” in which the vertical axis of the image represents time (27). Such 3-D renderings are particularly effective in displaying protracted

Figure 1. Confocal microscopy of fertilization-induced calcium dynamics. All images were obtained with a Bio-Rad® MRC-600 confocal laser scanning microscope (Bio-Rad Laboratories, Hercules, CA, USA) and subsequently rendered in a pseudocolored format in which low calcium levels are depicted as blues and higher calcium concentrations appear as greens and reds. (A) Fertilization-induced calcium wave (arrow) in an oocyte of the starfish *Pisaster ochraceus*. The oocyte was injected with Calcium Green Dextran (CG, 10 000 MW) and imaged at 5-s intervals while undergoing fertilization. Individual optical sections were then subjected to single-channel “ratioing” (i.e., F/F_0 where F_0 = the first image of the sequence and F = subsequent images) to correct for differential pathlength and dye loading artifacts. (B) Demonstration of dual-channel ratioing with a single wavelength calcium indicator. An oocyte of the nemertean worm *Cerebratulus lacteus* was injected with a mixture of Calcium Green plus the calcium-insensitive dye Rhodamine B Dextran™ (Rh) so that dual-channel images could be simultaneously collected and subsequently ratioed (CG/Rh) to correct for differential pathlength and dye loading artifacts. Note that before ratioing, the periphery of the spherical egg shows brighter fluorescence. This increased fluorescence is not caused by higher calcium levels, but rather to a shorter pathlength and less quenching of the fluorescent signal than occurs in the central region of the egg. The CG/Rh ratios help to compensate for such differential pathlength artifacts. (C) Dual-channel ratioed images collected every 5 s from a fixed optical plane within an egg of the sea urchin *Lytechinus pictus*. The egg had been injected with a mixture of Calcium Green and Rhodamine before dual-channel ratioing. A fertilization-induced calcium wave (arrow) can be seen propagating across the entire ooplasm. (D) A 3-D rendering of a time-lapse confocal data set showing multiple calcium transients (arrows) in a fertilized oocyte of *C. lacteus*. The oocyte had been injected with Calcium Green and Rhodamine before dual-channel collection of confocal sections every 5 s from a fixed optical plane within the oocyte. The circular optical sections were then prepared as CG/Rh-ratioed images and stacked on top of each other to provide a cylinder whose vertical axis represented time. The cylinder was subsequently cleaved through its center to show the entire time-lapse data set. The top of the cleaved cylinder corresponds to the onset of imaging before fertilization, and the vertical scale bar represents 10 min. (E) A 2-D montage of CG/Rh-ratioed sections depicting two fertilization-induced calcium waves (arrows) arising from a common onset site in an oocyte of the nemertean worm *Emplectonema gracile*. Horizontal scale bars = 100 μ m.

sequences of calcium dynamics (Figure 1D). Moreover, the slope of the calcium transient in these outputs is indicative of the speed of wave propagation across the egg and can be used to demonstrate differences in transmission rates within the peripheral versus central regions of the egg (25). Besides monitoring the initial calcium response at fertilization, long-term CLSM analyses have been used to track changes in the spatial-temporal properties of intracellular calcium fluxes following fertilization (22). Such extended imaging studies have also been helpful in identifying repeated onset points or “pacemaker” sites (2,23) in the ooplasm of some zygotes (Figure 1E).

Where Might Future Studies Be Heading?

For future CLSM investigations, it seems likely that new calcium-sensitive fluorophores and lasers for exciting such fluorophores will aid analyses of fertilization-induced calcium dynamics. Moreover, a general improvement in the acquisition rates of confocal microscopes, as has been recently achieved in a few laboratories (6), should facilitate the compilation of extremely valuable data regarding the rapid “elementary events” of calcium elevations. These events generally precede the propagation of a global calcium wave in

various cell types that have been examined (28).

Not only are enhancements in temporal resolution to be expected but also further melding of confocal analyses with related technologies such as microscopy using multiphoton excitation should provide additional benefits. For example, it is clear that two-photon excitation methods can greatly increase the depth within the sample from which images can be obtained (1) and thus allow improved imaging of relatively large oocytes and eggs. Further benefits of multiphoton excitation include both the ability to reduce photobleaching by restricting the excitation events to the plane of focus and the reduction of specimen damage by using ultrashort pulses of relatively long wavelengths (19).

Finally, resolution achieved both in the lateral and vertical axes using either multi- or single-photon excitation can also be enhanced by advances in deconvolution methods that are applicable to confocal data sets. Such image processing reduces noise and clarifies signal within the image by mathematical means rather than by relying only on mechanical filtering by the confocal aperture (17). Thus, the deconvolved images might provide even more precise spatial information in relatively thick cells such as eggs than can be obtained by conventional CLSM.

Undoubtedly, there will be numerous advances in the field of CLSM imaging analyses. The exact nature of these improvements is difficult to predict accurately. However, regardless of what specific enhancements lie ahead, the future seems bright for both confocal microscopy as we know it today and for what it will eventually become.

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