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Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability

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Abstract

A major challenge for fluorescence imaging of living mammalian cells is maintaining viability following prolonged exposure to excitation illumination. We have monitored the dynamics of mitochondrial distribution in hamster embryos at frequent intervals over 24 h using two-photon microscopy (1,047 nm) while maintaining blastocyst, and even fetal, developmental competence. In contrast, confocal imaging for only 8 h inhibits development, even without fluorophore excitation. Photo-induced production of H₂O₂ may account, in part, for this inhibition. Thus, two-photon microscopy, but not confocal microscopy, has permitted long-term fluorescence observations of the dynamics of three-dimensional cytoarchitecture in highly photosensitive specimens such as mammalian embryos.

Keywords

two-photon microscopy; laser scanning confocal microscopy; live cell fluorescence imaging; embryo; mitochondrial dynamics; mammal; hamster

The detection of specific cellular components by imaging techniques such as wide-field epifluorescence or laser scanning confocal microscopy (LSCM) requires exposure to high intensity light that can cause cellular damage¹. Consequently, the quantity or quality of images that can be collected is limited or, even worse, the reliability of the images may be compromised. This is a particular problem when imaging events that occur over periods of time ranging from hours to days, such as embryonic development. For this reason, much of our current understanding of subcellular morphological changes during mammalian embryonic development is based on images of fixed or static specimens at different developmental stages^{2–6}. Thus, it can be difficult to interpret dynamic processes accurately, because the continuity of events must be inferred. The establishment of long-term fluorescence imaging methods that maintain the viability of live specimens is critical for advancing our understanding of cell biology and embryonic development in areas such as ion dynamics⁷, cytoplasmic reorganization, compaction and blastocoel formation, embryonic development in exotic species (where specimens are heterogeneous and difficult

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to obtain), use of fluorescent tags for the preselection of embryos for subsequent embryo transfer⁸, and studies of protein expression in living cells using green fluorescent protein⁹.

Embryos of some mammals, particular hamsters, are very sensitive to culture conditions¹⁰. Furthermore, studies suggest that mammalian oocytes and embryos are adversely affected by exposure to visible light^{11–13}. Because of this sensitivity, mammalian embryos are ideal to test live-cell imaging techniques. In addition, there are obvious morphological changes associated with differentiation, namely compaction and blastocoel formation, which can be used to assess viability. The embryo must undergo cell division during and after imaging as well as maintain a level of developmental competence that allows it to initiate differentiation.

Two-photon laser scanning microscopy (TPLSM) is a fluorescence imaging technique that has been promoted as possibly less harmful to living cells¹⁴. Two (or more) infrared photons simultaneously excite a fluorophore rather than a single visible photon^{14–16}. Therefore, the lower energy per photon of the longer wavelength, as well as the restriction of fluorophore excitation to the focal plane, should reduce the total photodamage to the specimen compared with conventional LSCM. However, it is still unclear whether TPLSM is a suitable technique for long-term imaging studies of living preparations because exposure to the very high intensity illumination of TPLSM may be damaging to living cells. It has been demonstrated that TPLSM mean excitation doses at levels above 6 mW and 150 fs at wavelengths of 800 nm can compromise tissue culture cell viability¹⁷. Furthermore, concerns have been raised that the longer wavelengths used for TPLSM may cause localized heating of the specimen^{18,19}.

Previous work using LSCM suggested a correlation between mitochondrial organization and developmental competence in cultured hamster embryos²⁰. Therefore, we compared directly the efficacy of 1,047 nm TPLSM (~1.2 eV photon energy) to conventional LSCM (~2.5 eV photon energy) for studying the dynamics of mitochondrial distribution in early hamster embryos, with emphasis on maintaining viability over long imaging periods. Here, we show that two-cell hamster embryos with fluorescently labeled mitochondria that are repetitively imaged by TPLSM are subsequently capable of blastocyst and even fetal development, whereas confocal imaged embryos never reach the morula stage. In addition, we have found that 514 nm LSCM imaged embryos exhibit an increase in oxidative stress during imaging, whereas TPLSM-imaged embryos do not, suggesting that the production of H₂O₂ may contribute to the reduced viability seen in embryos imaged with LSCM.

Results and discussion

LSCM inhibited development, whereas TPLSM maintained embryo viability

We have found that the development of hamster embryos was dramatically impaired by imaging with LSCM (flux density = 9×10^3 W/cm²; 8 μ s dwell time). None of the two-cell embryos (with fluorescently labeled mitochondria) imaged over 8 h (five optical sections collected every 15 min) with LSCM (total exposure = 280 μ J per embryo) developed to the morula or blastocyst stage (Fig. 1A–C and I). However, the nonimaged control embryos cultured at the same time in a separate drop in the same culture dish developed at least to the

morula stage (Fig. 1D and I), showing that the impaired development was due to the laser exposure rather than to effects of the culture conditions. Occasionally, embryos in the imaged sample were out of the field of view and therefore not directly subjected to the LSCM irradiation. Among this subset of embryos, all but one ($n = 12$) developed to at least the morula stage, indicating that the detrimental effect of imaging with these wavelengths occurs directly on the embryos rather than by altering the culture medium²¹. Development was inhibited by each of the three wavelengths (514, 532, and 568 nm) used to excite this fluorophore. Many of these LSCM-imaged embryos were unable to undergo even a single division ($n = 14$ embryos: two-cell = 50%; three- to four-cell = 29%; five- to seven-cell = 7%; eight-cell = 14%). Even more remarkable is the fact that the development of *unstained* embryos was also inhibited under identical excitation conditions (Fig. 1I) ($n = 22$ embryos: two-cell = 48%; three- to four-cell = 30%; five- to seven-cell = 13%; eight-cell = 9%), suggesting that these wavelengths can also directly affect the embryos rather than simply causing damage indirectly via excitation of the fluorophore.

In striking contrast, embryo viability is maintained when embryos are imaged using the same microscope system with a 1,047 nm ultrashort pulsed laser (flux density = 6×10^6 W/cm²; 8 μ s dwell time). We found that stained embryos imaged for 24 h (five optical sections every 15 min for a total exposure of ~ 2 J per embryo) developed to morulae and blastocysts (Fig. 1E–G) in proportions that were not significantly different from the nonimaged controls (Fig. 1I: imaged = 0.90 ± 0.16 ; nonimaged = 0.95 ± 0.13 ; $p = 0.6$).

To establish whether increasing the frequency of data capture would affect development, we imaged two-cell embryos every 2.5 min for 24 h. These embryos also developed to morulae and blastocysts in proportions that were not significantly different from their nonimaged controls (Fig. 1I; imaged = 0.89 ± 0.15 ; nonimaged = 0.83 ± 0.24 ; $p = 0.42$). This maintenance of viability with TPLSM is particularly impressive because, compared with the LSCM-imaged embryos, these stained, TPLSM-imaged embryos received 42,860 times more total irradiation exposure: 2,381 times greater dose per image (4.16 mJ versus 1.75 μ J) and an 18-fold increase in the number of images collected (2.5 min versus 15 min interval between image collection and 24 h versus 8 h). Furthermore, these embryos received approximately 1,000 times the average laser power (13 mW versus 10–30 μ W) at a wavelength with much greater potential for sample heating due to the absorption spectrum of water. Thus, for a laser scanning system imaging optically transparent embryos, the potential heating due to water absorption is not a factor limiting viability. This viability comparison between the 1,047 nm laser and the 514 nm, 532 nm, and 568 nm LSCM laser lines indicates that the infrared wavelength is considerably more benign to mammalian embryos.

Peroxide is produced in LSCM-imaged embryos

As demonstrated above, embryos imaged with the three LSCM wavelengths never reached the morula or blastocyst stages and typically arrested with no, or only one, division. One possible explanation for the observed developmental arrest is the generation of free radicals from the excited fluorophore, which may damage cellular components¹. However, the results presented here clearly demonstrate that this cannot be the sole explanation for the developmental arrest of LSCM-irradiated embryos because embryo viability is impaired

even in the absence of fluorophore (Fig. 1I). Our results also show that the arrest is not due to alterations in the culture medium, as unimaged embryos in the same drop remain viable. Cellular damage caused by light has been shown in other cell types for various wavelengths, ranging from 295 to 500 nm (one photon)^{22,23} or 730 nm to 800 nm (two photons)¹⁷. Therefore, we sought to determine a possible endogenous source of this phototoxicity.

One potential source of toxicity is the production of hydrogen peroxide or other reactive oxygen compounds, which have been implicated in contributing to the developmental arrest of cultured embryos at the two-cell stage from some strains of mice^{24,25} and following visible light exposure in hamster embryos²⁶. The formation of hydrogen peroxide has been observed in other cell types in response to higher energy light, such as that which occurs with epifluorescence. To determine whether embryos exhibit a similar response to irradiation, we utilized a derivative of dihydrofluorescein (DHF), a dye that fluoresces in the presence of reactive oxygen^{27–30}. Embryos exposed for 5 s to irradiation with epifluorescence (Fig. 2A, F, and K) showed a response similar to that demonstrated in tissue culture cells^{23,31}, namely that DHF fluorescence increased following irradiation. Interestingly, the pattern of fluorescence mirrors that of the mitochondrial distribution (compare Fig. 2F with Fig. 1A and E), supporting the proposal that mitochondria are the initial, and possibly the major, source of reactive oxygen in response to damaging light^{17,23,31,32}. This increase was not due to endogenous autofluorescence, as unstained embryos do not exhibit this increase after irradiation (Fig. 2C, H, and K). Because DHF is thought to be primarily sensitive to the production of hydrogen peroxide^{29,33}, we determined if this was also the case in these epifluorescent-irradiated embryos. When embryos were pretreated with catalase to promote the degradation of hydrogen peroxide, the increase in DHF fluorescence was attenuated compared with embryos not treated with catalase (Fig. 2B, G, and K). Although this reduction in fluorescence was only marginally significant ($p = 0.09$), it does suggest that these embryos were making some hydrogen peroxide in response to 450–490 nm epifluorescence illumination. These results support and extend the results reported for cultured mammalian cells²³.

To determine if hydrogen peroxide is also produced in LSCM-irradiated embryos, thereby possibly contributing to reduced viability, we compared the pre- and post-irradiation DHF fluorescence intensity of embryos imaged for 8 h with either LSCM (514 nm) or TPLSM under an imaging regimen similar to that used for the developmental competence studies (five optical sections, 5 μ m apart, every 15 min). Embryos imaged with the 1,047 nm laser showed little change in DHF fluorescence (Fig. 2D, I, and K), and the change was not different from that observed in their nonimaged controls ($p = 0.45$). However, those embryos imaged with the 514 nm laser showed a dramatic increase (Fig. 2E, J, and K), which was significantly different from their nonimaged controls ($p = 0.001$). This suggests that the reduction in viability seen in embryos irradiated with 514 nm LSCM is partly due to photoinduced peroxide production, whereas embryos imaged with 1,047 nm TPLSM are not subjected to the same levels of oxidative stress.

TPLSM also maintains developmental competence

To determine whether imaged embryos were capable of subsequent development, we imaged mitochondria-labeled embryos as described above with TPLSM (Fig. 3A–C) and, at 82 h post-egg activation (PEA) (Fig. 3D), transferred the imaged embryos into recipient females. We transferred a total of eight imaged, golden hamster embryos into four successfully pseudopregnant females and recovered three near-term golden hamster fetuses (Fig. 3E) at two days preparturition, as well as one live golden hamster pup from a full-term pregnancy. This fetal return rate (50.0%) is not significantly different from nonimaged control embryos cultured on the microscope stage (72.7%: $n = 11$ embryos transferred, $p = 0.3$) or the cotransferred albino helper embryos (45.8%: $n = 24$ embryos transferred, $p = 0.8$). Furthermore, the live offspring obtained from one of the stained, imaged embryos has given birth to 13 apparently normal pups, indicating that the imaging has not impaired her reproductive capabilities. Therefore, the blastocysts developed from TPLSM-imaged embryos seem to maintain developmental competence, namely the capacity to produce apparently normal, viable offspring. Not only does this maintenance of developmental competence validate the minimally invasive nature of this technique but also holds great promise for the use of 1,047 nm TPLSM with yellow fluorescent protein-tagged transgenic embryos, as well as the use of other fluorescent markers, for pretransfer embryo screening.

These studies show that 514, 532, or 568 nm light, focused by a high numerical aperture lens, can be intrinsically damaging to live mammalian embryos, severely restricting the use of LSCM for long-term developmental analyses. Some of the cellular damage was probably due to the production of hydrogen peroxide during LSCM illumination of the embryos. More importantly, we show that near-infrared TPLSM generates high-resolution images without measurably affecting developmental potential, even with longer and more frequent image collection. This is particularly important for studies that require the maintenance of viability in order to correlate sequential events in individual specimens. This clear demonstration that 1,047 nm TPLSM phototoxicity is below the threshold of perceptible damage for living mammalian embryos opens the door for a multitude of long-term live imaging studies.

Experimental protocol

Embryo collection and preparation

Two-cell embryos were collected from hamster (*Mesocricetus auratus*) females between 35 and 37 h PEA. Hamsters have a consistent reproductive cycle such that embryos collected at the same time of day are developmentally similar, with the majority of embryos in interphase at the initiation of imaging. The embryos were stained for 15 min with 330 nM Mitotracker-X-Rosamine (Molecular Probes, Eugene, OR)³⁴, rinsed, and cultured on the microscope stage^{34,35} in 36 μ l drops of hamster embryo culture medium-9 (HECM-9)³⁶. An environmental chamber surrounding the microscope was heated to maintain embryos at approximately 37.5°C and a mini-chamber on the stage maintained gases at 10% CO₂ and 5% O₂. After imaging, embryos were returned to the incubator, and developmental stage was determined at 82–84 h PEA.

Image collection

For both LSCM and TPLSM, each image was collected as a single slow scan (SOM software; Bio-Rad, Hercules, CA) with a Nikon (Melville, NY) 40× plan fluor oil-immersion 1.3 numerical-aperture objective lens. At each time point, a z-series of five optical sections, 5 μm apart, was collected. Imaging for 8 h included one cell division, while 24 h of imaging included two cell divisions. Statistical comparison between imaged and nonimaged TPLSM embryo development was performed using a paired Student's *t*-test. Embryo images were arranged as figures in Adobe (San Jose, CA) PhotoShop 4.0. All adjustments in figure quality for publication were performed on the entire set of images to maintain relative image quality and information.

Microscopy

The LSCM used was a Bio-Rad 600 scan head and one of the following excitation lasers: 514 nm argon (power = 12 μW); 532 nm ADLAS 300 (Advanced Design Lasers, Laser Lines, Banbury, UK) doubled neodymium-doped:yttrium aluminum garnet (Nd:YAG ; power = 10 μW); or 568 nm krypton/argon (power = 28 μW). The 514 nm laser was used to maximize the emission collection, 532 nm was used because it is close to the effective excitation (523 nm) of the 1,047 nm laser, and 568 nm was used because it is close to the excitation peak of Mitotracker-X-Rosamine. Neutral density filters (0.1 optical density steps) were set to generate an initial, single optical section of comparable average intensity per voxel as those obtained with TPLSM. The aperture was set to generate optical section thickness (1.9 μm) comparable to that obtained with TPLSM. For LSCM, the focused beam slightly underfilled each pixel (about 65%). The TPLSM was designed and used at the Integrated Microscopy Resource at the University of Wisconsin–Madison. It has been described in detail^{16,37}. Briefly, this system uses a solid-state, diode-pumped, neodymium-doped:yttrium lithium fluoride-based (Nd:YLF) laser (DPM 1000-PC, Microlase Optical Systems, Glasgow, UK) with a fixed wavelength of 1,047 nm, a maximum mean power of 550 mW, and a pulse duration of 175 fs. At the point of focus, the effective excitation wavelength is near 523 nm. The measured optical section was 1.9 μm (underfilled back aperture), and the focused beam slightly overfilled each pixel (approximately 129%).

Determination of peroxide production

Two-cell embryos were stained with 37 μM 6-carboxy-2'7'-dichlorodihydrofluorescein di(acetyloxymethyl ester) (DHF; dihydrofluorescein; Molecular Probes)²³ in HECM-9³⁶ for 15 min under culture conditions, rinsed twice, then placed in culture drops. All fluorescence intensity measurements were determined on single optical sections (approximately midsagittal) such that the pre- and post-irradiation comparisons were performed on the same, or similar, optical section. For epifluorescent irradiation, individual embryos were placed in 5 μl drops, imaged with TPLSM, irradiated for 5 s with 450–490 nm epifluorescent irradiation, then imaged again with TPLSM. The fluorescence increase was expressed as the ratio of the post-irradiation to the pre-irradiation fluorescence intensity of each blastomere in the TPLSM images. Catalase-treated embryos were placed in 1,700 U/ml catalase (Sigma, St. Louis, MO) in HECM-9 for 2.5 h before staining with DHF, rinsed and imaged. For each TPLSM replicate, three DHF-stained embryos were placed in each of two 30-μl drops, and

embryos in one drop were imaged as described (five optical sections, 5 μ m apart, every 15 min) for 8 h. The relative fluorescence value for the imaged embryos was the ratio of the pixel intensity of each blastomere at $t = 8$ h to their $t = 0$ values. The relative fluorescence value of the nonimaged embryos was the ratio of the average pixel intensity of all of the blastomeres at $t = 8$ h to the average pixel intensity of the imaged blastomeres at $t = 0$. For LSCM experiments, embryos were irradiated every 15 min (five optical sections, 5 μ m apart) for 8 h with 514 nm excitation. Relative fluorescence was calculated from TPLSM images collected before and following LSCM imaging. These relative fluorescence intensity values were statistically compared with those of the nonimaged controls using an unpaired two-sample t -test. To confirm the presence and activity of the dye, embryos in a separate drop were tested for a fluorescence increase following epifluorescent irradiation immediately before and following each TPLSM or LSCM experiment.

Embryo transfer

Embryos were collected, stained, and imaged or cultured as described, except that the culture medium included 5 mM glucose to improve fetal development³⁸. Albino embryos, cultured from the two-cell stage, were cotransferred to improve pregnancy maintenance. One to three golden hamster embryos and six albino embryos were transferred, at 82 h PEA, into the left uterine horn of a -1 day asynchronous pseudopregnant recipient female. Fetuses were removed two days prepartum and eye color was recorded, or pregnancy was allowed to go to term. The recovered black-eyed, golden hamster fetuses or offspring could not have been the result of a natural pregnancy of the recipient female because (1) the vasectomized males used for mating were known to be sterile and (2) no implantation sites were detected in the right uterine horn. Fetal return rates were compared using a χ^2 test.

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Figure 1. Viability of embryos after long-term imaging. 514 nm LSCM (A and B) or 1,047 nm TPLSM (E and F) single optical sections of Mitotracker-labeled embryos at the start (A and E) and the end (B and F) (t = 8 h in B and t = 24 h in F) of an imaging sequence. Nomarski images of the TPLSM-imaged embryos (C and G) and their nonimaged stage controls (D and H) at the time of expected blastocoel formation (82 h PEA). (I) Graph depicts the percentage of embryos per replicate that developed to morulae and blastocysts following imaging under various conditions. Values above columns represent number of embryos, and

each pair of columns represents at least three replicates. Imaging period is the total time over which the embryos were imaged. Embryos were either stained (+) or not stained (–) with mitochondrial label. Imaging frequency indicates time interval between z-series collection in minutes. Error bars represent one standard deviation from the mean of the replicates. Scale bar = 45 μm .

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Figure 2.

Production of reactive oxygen in imaged embryos. Two photon images of two-cell embryos (A–E) before and (F–J) following irradiation with: (F–H) 450–490 nm epifluorescence (5 s irradiation); (I) 1,047 nm TPLSM (every 15 min for 8 h); or (J) 514 nm LSCM (every 15 min for 8 h). All embryos, except (C) and (H), were labeled with DHF. Embryo in (B) and (G) was treated with catalase before staining and irradiation. (K) Quantitation of change in fluorescence intensity. Bars indicate the mean post-irradiation fluorescence of blastomeres relative to each blastomere's fluorescence before irradiation. Values above bars are numbers of blastomeres analyzed. Error bars represent one standard deviation of the mean blastomere intensity value. Scale bar = 45 μ m.

Figure 3.

Fetal development following long-term imaging. Two photon images of mitochondria-labeled embryos at (A) the initiation of imaging (B) after 8.5 h of imaging, showing a mitotic spindle (arrow), and (C) the completion of a 24 h imaging sequence. (D) After imaging, embryos were cultured in the incubator until 82 h PEA (Nomarski) at which point they were transferred to a recipient female. (E) A black-eyed fetus that developed from one of these imaged embryos is shown next to an albino uterine mate. Scale bar for (A–D) = 45 μ m. Scale bar for (E) = 4.75 mm.