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Following the course of pre-implantation embryo patterning by non-linear microscopy

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ABSTRACT

Embryo patterning is subject to intense investigation. So far only large, microscopically obvious structures like polar body, cleavage furrow, pro-nucleus shape can be evaluated in the intact embryo. Using non-linear microscopic techniques, the present work describes new methodologies to evaluate pre-implantation mouse embryo patterning. Third Harmonic Generation (THG) imaging, by detecting mitochondrial/lipid body structures, could provide valuable and complementary information as to the energetic status of pre-implantation embryos, time evolution of different developmental stages, embryo polarization prior to mitotic division and blastomere equivalence. Quantification of THG imaging detected highest signalling in the 2-cell stage embryos, while evaluating a 12–18% difference between blastomeres at the 8-cell stage embryos. Such a methodology provides novel, non-intrusive imaging assays to follow up intracellular structural patterning associated with the energetic status of a developing embryo, which could be successfully used for embryo selection during the *in vitro* fertilization process.

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1. Introduction

Although in most species embryo polarity is apparent from the early developmental stages, including spatial patterning of the egg, for many years mammals were believed to acquire embryonic polarity shortly after gastrulation, during the interaction with maternal uterus for implantation (Beddington and Robertson, 1998). The main reasons for that belief included first the fact that body axes in the mouse embryo become morphologically apparent during gastrulation. A second reason was based on the absence or yet un-identified programmed distribution of morphogenetic determinants from the mammalian embryo, which could be determined in *Caenorhabditis elegans, xenopus, zebrafish*, and thirdly the plasticity the mammalian embryo may display. Despite the imposed experimental perturbation disturbing early patterning, early mouse embryos may continue their development. However, embryo plasticity during the early pre-implantation stages does not exclude polarity. Indeed, polarity is apparent since the second mitotic division, where the polar body is a landmark for the bilateral symmetry of the blastocyst (Ciemerych et al., 2000). Embryonic polarity is also related to the position of sperm entry (SEP; Piotrowska and Zernicka-Goetz, 2001) which is located close to the cleavage furrow at the surface of one of the blastomeres.

However, except from the classical microscopically observed structures, including the polar body, SEP, cleavage furrow, pronucleus shape, in order to study embryo patterning one should be able to detect intracellular structures of the intact pre-implantation embryo and follow up development in a non-invasive manner. To this extend, the present work attempted to evaluate pre-implantation embryo patterning and polarity using the non-linear (Two Photon Excitation Fluorescence (TPEF) and Second and Third Harmonic Generation (SHG, and THG)) imaging techniques. So far, by employing these non-linear imaging modalities, valuable and complementary information, from various biological samples at microscopic level, have been extracted (Campagnola and Loew, 2003; Filippidis et al., 2009; Zipfel et al., 2003). These non-destructive techniques represent the forefront of research in cell biology since they have the potential to offer new insights into complex developmental processes. In addition, they can probe morphological changes and elucidate functions of tissues and individual cells in many biological settings.

Abbreviations: THG, Third Harmonic Generation; SHG, second harmonic generation; TPEF, Two Photon Excitation Fluorescence; FRET, fluorescence resonance energy transfer; hCG, human chorionic gonadotropin; PMSG, pregnant mare serum gonadotropin.

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Third-harmonic generation process represents a non-linear scattering phenomenon. Upon interaction of intense laser radiation with matter, three photons of angular frequency ω are destroyed and a photon of angular frequency 3ω is simultaneously created in a single-quantum mechanical process (Barad et al., 1997). THG is sensitive to local differences in third-order non-linear susceptibility $\chi^{(3)}$, refractive index and dispersion (Debarre and Beaurepaire, 2007). In THG imaging microscopy, the contrast arising from interfaces, changes in the refractive index values and optical heterogeneities of size comparable to the beam focus. Therefore, THG imaging modality can be used as a powerful, non-destructive diagnostic tool providing unique structural, anatomical and morphological information in various biological samples at cellular and sub-cellular level. The main contributors of the high THG signals from cells are mitochondria (Hsieh et al., 2008) and lipid bodies (Debarre et al., 2006; Watanabe et al., 2010). The use of ultra-short pulse lasers (pulse duration in the femtosecond timescale) allows THG at low energies, which ensure cell viability.

In embryology, several imaging techniques, such as confocal laser scanning microscopy (Cockell et al., 2004) and differential interference contrast (Hamahashi et al., 2005) have been employed to track cell division stage of biological specimens. Recently, the non-linear imaging modality of third harmonic generation has been used, as an alternative method, providing information related to morphological changes and complex developmental processes of *Zebrafish* (Chen et al., 2006; Chu et al., 2003; Sun et al., 2004), *Drosophila* (Debarre et al., 2006; Supatto et al., 2005), *Xenopus laevis* (Oron et al., 2004), *C. elegans* (Tserevelakis et al., 2011; Aviles-Espinosa et al., 2010) and mouse (Jesacher et al., 2009; Watanabe et al., 2010) embryos. THG microscopy presents several advantages compared with other microscopic techniques, such as the capability of intrinsic three-dimensionality, the ability to section deep within tissues and the reduction of "out of focal plane" photobleaching and phototoxicity in the biological specimens. Furthermore, optical higher harmonic generation, including THG, does not deposit energy to the specimen due to its energy-conservation characteristics, providing minimal sample disturbance which is desirable for the biological studies in vivo. By employing THG as a microscopic contrast mechanism for embryogenesis studies, minimum preparation and no staining of the samples are required. In addition, cellular and sub-cellular processes can be monitored for a prolonged period of time.

Using THG imaging, the aim of the present work was to study pre-implantation embryo patterning and polarity during the first developmental stages and evaluate whether such recording could predict embryo's next move, providing thus useful histological information for further exploitation.

2. Materials and methods

2.1. Animals

BALB/c mice were purchased from Charles River (Milan, Italy), maintained in the animal facility at the University of Crete (Crete, Greece) and their care was in accord with the Institution's guidelines. Four to 10 weeks old males and females were used in all performed experimentations.

2.2. Embryo collection and manipulation upon in vivo fertilization

BALB/c female mice were superovulated by intraperitoneal injection of pregnant mare serum (PMSG, 5 IU, Sigma, St. Louis, MO, G4877) and human chorionic gonadotropin (hCG, 5 IU, Sigma, CG5–1VL) at an interval of 47 h. Superovulated females were placed with male breeders 2 h post-hCG injection and remained caged together overnight. Next morning, mating was detected by the presence of a vaginal plug. Embryos were collected from dissected oviducts or uteri using pre-warm KSOM-AA medium (EmbryoMax®KSOM, Millipore, Billerica, MA, MR-121-D) covered with mineral oil (Sigma, M-8410) and were cultured in 50 μl drops of pre-warmed KSOM-AA medium (maximum 5 embryos per drop) covered with mineral oil, at 37 °C in a 5% CO_2 atmosphere. Depending on the desired embryo stage, females were sacrificed at the appropriate time post-hCG; zygotes, 18 h; 2-cell, 44 h; 4-cell, 60 h; 8-cell, 72 h; early morula, 78 h; early blastocyst, 96 h. 4- and 8-cell embryos were also disaggregated to single blastomeres after the removal of zona pellucida using pre-warmed acidic Tyrode's solution (Sigma, T1788) for 10–15 s.

2.3. Immunocytochemistry and confocal microscopy

Embryos were stained with TOPRO-3 (TOPRO-3®, 1:1000 dilution, Molecular Probes, T3605) using the fix and perm kit (FIX & PERM® cell permeabilization kit, Invitrogen, CA, USA, GAS-003). Upon staining, embryos were transferred in 25% glycerol and processed to confocal microscopy analysis (Leika, Solms, Germany).

2.4. Third harmonic generating apparatus

The use of femtosecond (fs) lasers enables high peak powers for efficient non-linear excitation, but at low enough energies, so that the biological samples are not damaged. In this study, an amplitude system femtosecond *t*-pulse laser (1028 nm, 50 MHz, 1 W, 200 fs) has been employed as an excitation source. Due to the advantage of the femtosecond laser system, emitting at 1028 nm, to generate a third harmonic signal in the near ultraviolet (UV) part of the spectrum (~343 nm), it was here applied for the realization of the non-linear measurements. Consequently, there is no need to use UV optics with special coatings for the collection of the THG signal. Moreover, by employing this excitation wavelength (1028 nm) the absorption of the water, which leads to unwanted thermal heating of the biological samples, is constrained.

The laser beam was guided into a proper modified Nikon upright microscope (Eclipse ME600D; Fig. 1). THG signals were detected in the forward direction. A 32×, 0.85 numerical aperture (NA) objective lens (Carl Zeiss, C Achroplan, water immersion) was employed for tight focusing of the laser beam onto the sample and a 100×, 1.4 NA condenser lens (Carl Zeiss, Plan Apochromat, oil immersion) was used for recording THG signals. The scanning procedure was performed with a pair of galvanometric mirrors (Cambridge Tech. 6210H) and the focal plane was selected with a motorized translation stage (Standa 8MT167-100, 1 μm minimum step). Lab View interface controlled both scanning and data acquisition procedures. A CCD camera (PixeLINK PL A662) was used for observation of the specimens (Fig. 1). Mouse embryos were placed between two very thin (~70 μm) round glass slides (Marienfeld). The glass slides were separated with a 100 μm thick spacer in order to avoid damaging the samples. After filtering with a 340 nm color glass filter (Hoya U 340), THG was detected by a photomultiplier tube (PMT Hamamatsu H9305-04). The average laser power on the specimen was 20 mW (0.4 nJ per pulse).

A single two-dimensional (2D) THG image of 500 × 500 pixels was recorded in less than one (1) second. In order to improve the signal to noise ratio one 2D optical section was made of thirty (30) averaged scans. The time for obtaining a slice image, was thirty (30) seconds. To create a three-dimensional (3D) reconstruction of our specimen, a series of 2D optical sections separated 2 μm apart were acquired. The whole procedure of data acquisition needed for the 3D reconstruction of THG images from mouse

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