Light-sheet microscopy for quantitative ovarian folliculometry

Hsiao-Chun Amy Lin[a,b+] , Rahul Dutta[c+] , Subhamoy Mandal[a,d] , Alexander Kind[c], Angelika Schnieke[c], Daniel Razansky[a,b*]

[a]Institute for Biological and Medical Imaging, Helmholtz Zentrum München, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany; [b]Faculty of Medicine, Technische Universität München, Ismaningerstraße 22, 81675 Munich, Germany; [c]Chair of Livestock Biotechnology, Technische Universität München, Liesel-Beckmann Straße 1, 85354 Freising, Germany; [d]Chair for Biological Imaging, Faculty of Electrical Engineering and Information Technology, Technische Universität München, Arcisstraße 21, 80333 Munich, Germany

[+]equal contribution * Corresponding author: dr@tum.de; phone +49 89 3187 1587; fax +49 89 3187 3017

ABSTRACT

Determination of ovarian status and follicle monitoring are common methods of diagnosing female infertility. We evaluated the suitability of selective plane illumination microscopy (SPIM) for the study of ovarian follicles. Owing to the large field of view and fast acquisition speed of our newly developed SPIM system, volumetric image stacks from entire intact samples of pig ovaries have been rendered demonstrating clearly discernible follicular features like follicle diameters (70 μm - 2.5 mm), size of developing Cumulus oophorus complexes (COC) (40 μm - 110 μm), and follicular wall thicknesses (90 μm-120 μm). The observation of clearly distinguishable COCs protruding into the follicular antrum was also shown possible, and correlation with the developmental stage of the follicles was determined. Follicles of all developmental stages were identified, and even the small primordial follicle clusters forming the egg nest could be observed. The ability of the system to non-destructively generate sub-cellular resolution 3D images of developing follicles, with excellent image contrast and high throughput capacity compared to conventional histology, suggests that it can be used to monitor follicular development and identify structural abnormalities indicative of ovarian ailments. Accurate folliculometric measurements provided by SPIM images can immensely help the understanding of ovarian physiology and provide important information for the proper management of ovarian diseases.

Keywords: Selective-plane illumination microscopy, Ovary, Follicle, Cumulus oophorus complexes, Folliculometry

1. INTRODUCTION

In recent years, owing to the ever-increasing level of environmental pollution and drastic lifestyle changes, the risk of ovarian ailments like premature ovarian failure (POF) and polycystic ovary syndrome (PCOS) are constantly on the rise. Altogether they constitute a major cause of female subfertility in the modern world. Determination of ovarian status and follicle monitoring constitute the first step in the evaluation of an infertile woman, thus making ovarian imaging the most common diagnostic approach of female infertility. The ovary is imaged for its morphology (normal or polycystic), for its abnormalities (cyst, dermoids, endometriomas, tumors etc.), for its follicular growth in ovulation monitoring, for evidence of ovulation and corpus luteum formation and function [1]. Newer advancements in diagnostic imaging technologies like pelvic MRI and ultrasound have provided new insights into the human reproductive system. Yet, the imaging contrast and spatial resolution of the clinical imaging methods are far inferior to those routinely attained with optical microscopy. Thus, histological evaluation of ex vivo samples remains of indispensable value for diagnostic and research purposes. However, the mainstream ex vivo study of ovarian follicles with histology imposes depth limitation inherent in traditional microscopy. In addition, physical sectioning is labor and time intensive while fixation and sectioning processes may further destroy some key morphological features in the studied samples.

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The size of the ovarian follicle, the size of the developing oocyte within, and the thickness of the follicular wall are all very important folliculometric parameters for clinical management of ovarian ailments including infertility cases. A normal mammalian ovary harbors 25,000-106 primordial follicles at puberty. A sketch illustrating the various stages is shown in Figure 1. Primordial follicle is the earliest stage of follicular growth. In primordial follicles, the oocyte is arrested in the last stage of prophase. At this stage, it is surrounded by a single layer of granulosa cells. When the primordial follicle receives hormonal stimulation, primary follicle is formed. The oocyte enlarges, and the follicular cells divide. A follicle that has two layers of follicular cells is called a primary follicle. During the follicular phase, a small cohort of follicles (approximately 10–14) begins to develop. One member of this group is physiologically selected to ovulate in uniparous mammals, exhibiting greatly increased hormonal activity and increasing its growth [2]. The others, lacking hormonal support, undergo atresia. The primary follicle makes a transition through the secondary follicle stage to develop into a Graafian follicle. In the Graafian follicle, the first meiotic division is completed, and the oocyte becomes a secondary oocyte. Upon ovulation, the oocyte begins its second meiotic division, as far as metaphase II. Further development only carries on if the ovum is fertilized. It is now known that follicles, which are destined to ovulate, display different image characteristics than those destined to atresia and that these characteristics are observable days before physiologic selection occurs. Identification of the follicle wall is useful in the characterization of the follicles of a cohort because the walls of follicles that are destined to ovulate are thinner [3]. With the commonly applied ultrasonography evaluations, the growing follicle looks just like a black bubble on the scanner without providing much folliculometric information. In ultrasound, most of the folliculometric information, including follicular wall thickness, is determined by manual or semi-automated segmentation.

The study here was undertaken to explore selective plane illumination microscopy (SPIM) as an imaging technique for attaining more accurate and informative folliculometric data from the scanned ovary. SPIM is a relatively new addition to the existing array of optical microscopy technologies which has significantly grown in recent years due to its ability for rapid visualization of both chemically-cleared and living specimen [4, 5]. In this imaging modality, a single plane of the sample is optically excited by a thin light sheet. This allows 2D fluorescent images to be captured by placing a camera orthogonal to the excitation light sheet. By translating either the light-sheet or the sample, 3D volumetric imaging stacks can be obtained. The method of optical sectioning rather than physical sectioning leaves the sample intact after imaging, which allows the same sample to be imaged from multiple views, which dramatically enhances the quality of the combined 3D rendering. Any reduction of the image quality due to physical destruction of the tissue is also avoided. SPIM thus offers a time- and labor-efficient way of imaging whilst maintaining high spatial resolution, and is a suitable method for producing high resolution imaging stacks of a 3D biological sample. To this end, this technique has been used for in-vivo studies of naturally transparent biological specimens, such as zebrafish and drosophilae pupae [6, 7], as well as imaging of diffuse samples in which scattering is removed by a chemical clearing procedure that matches the refractive index of the tissue to that of proteins, rendering the sample to be optically transparent [8, 9]. It was hypothesized that it may prove to be useful as an alternative tool for ovarian studies.

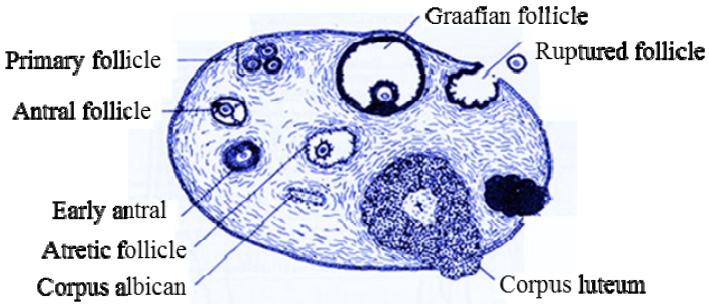

Figure 1. Sketch showing anatomy of the ovarian follicle. Primordial follicles are small, and usually found close to the outer edge of the ovarian cortex surrounded by a single layer of granulosa cells. When the primordial follicle receives hormonal stimulation, primary follicle is formed with two layers of granulosa cells. The primary follicle makes a transition through the secondary follicle stage where small accumulations of fluid in the intracellular spaces occur. These gradually coalesce to form an antrum and later the Graafian follicle is formed. In uniparous mammals one of the Graafian follicle, ovulate and the rest degenerate into Atretic follicles .Upon pregnancy the ovulating follicle forms the corpus luteum.

2. METHOD

The state of the art SPIM system used to image the ovarian follicle was built in-house. The light source was an 80 mW continuous wave DPSS laser at 670 nm (Frankfurt laser company, Germany), with a beam M2 of 1.10. A 5X telescope system was used to expand the beam diameter to 10 mm to allow excitation of the full width of the sample. A beam splitter was used to divide the main beam into two to allow double sided illumination to reduce effects of attenuation due to scattering in large samples.

A side-view sketch of the SPIM system concept is illustrated in Fig. 2a. Thin sheets of light were generated using cylindrical lenses with focal lengths of 40 mm. The light-sheets were oriented horizontally, with the sample centered at their beam waists. To capture 2D fluorescent images, a 5-MP scientific complementary metal oxide semiconductor camera (pco.edge, PCO, Germany) was placed directly above the sample, facing downwards. The imaging objective was an EC Epiplan-Neofluar objective (Zeiss, Germany). With a magnification of 2.5X and a NA of 0.06, it had a large working distance of 35 mm, and a depth of field of 220 µm, making it suitable for this application. The fluorescence signals were filtered using a 690/10 fluorescence filter (Chroma, USA), and a tube lens was used to relay the fluorescent image to the camera. The imaging system was characterized using line pair targets, and was determined to have a FOV of 10X9 mm with 6 µm lateral resolution. The axial resolution was characterized to be 17 µm at the beam waist, and increases to 60 µm at a 3 mm distance from the center of the FOV.

Slaughterhouse ovaries were brought to the laboratory within one hour of collection in a temperature-controlled box maintained at a temperature of 39o C. Individual Follicles with a diameter of 3-8mm and small groups of follicles were dissected out using ultrafine surgical blades. Immediately after follicle isolation they were stained with BCB, followed by chemical clearing. An alcohol based clearing method was used in the study [8, 9]. The dehydration process was performed by using 50%, 80%, and 100% ethanol for 12 hours each, repeating the last step twice. The dehydration step was followed by clearing of the follicles in 2 part benzyl alcohol and 1 part benzyl benzoate (BABB) solution. After 6 hours of clearing treatment the samples became transparent and were ready for imaging. Fig. 2b and 2c show the sample before and after clearing.

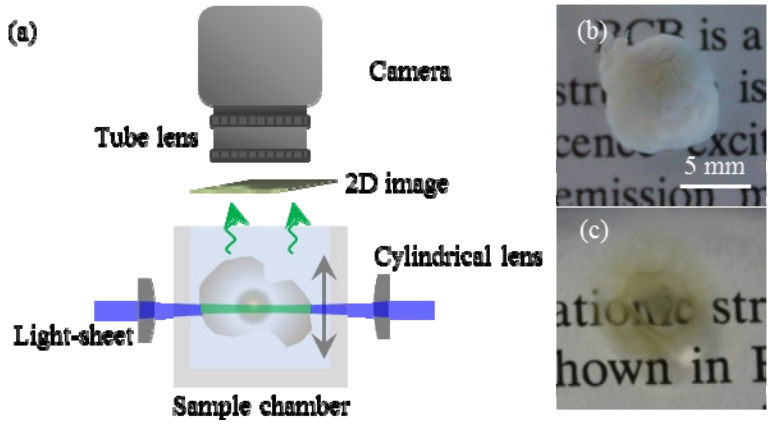

Figure 2. Experimental method. (a) Side view schematic of the developed fast SPIM imaging system. Two light-sheets were spatially overlapped and oriented horizontally. The imaging camera and the imaging optics were placed directly above the sample, which was centrally oriented at the beam waists of the light sheets. By placing the camera orthogonal to the light sheets, 2D fluorescent images through entire specimen measuring up to 10mm across could be captured. The samples were placed inside a glass chamber filled with clearing solution and scanned in a linear (vertical) geometry. By translating either the light-sheet or the sample chamber, full 3D image stacks of the sample could be obtained. (b) BCB stained ovarian follicle. (c) Sample after clearing with BABB.

During SPIM imaging, the samples were fixed on a holder and placed inside the sample chamber. A linear motorized stage (Thorlabs, USA) was used to translate the sample in a vertical motion, allowing different planes of the sample to be excited by the light-sheet. By translating the entire sample through the light-sheet, a complete stack of 2D SPIM images of the sample could be obtained. The cleared samples were imaged with the described SPIM system. The sample

chamber was filled with the clearing solution to match the refractive index of the medium to that of the sample, which avoids any image distortion due to refraction. Glass was specifically selected as the material for the sample chamber as it does not react with BABB, and maintained its clarity throughout imaging sessions. Analysis to extract morphological measurements were done using the software ImageJ.

3. RESULTS AND DISCUSSION

Ovarian histology broadly comprises of ovarian cortex and medulla. The outer cortex consists of ovarian follicles interspersed by stroma. The follicles are composed of cumulus oophorus, membrana granulosa, corona radiata, and the developing oocyte surrounded by zona pellucida. SPIM imaging results of cleared ovarian follicles are shown in Fig 3. The samples were able to be imaged in their entirety as their sizes were smaller than the camera FOV. A wide of range singular follicles were extracted individually from two different sets of image stacks. Fig 3a are single-slice images taken at two different planes of the same sample. The images demonstrate the high contrast (SNR) and image resolution achievable with SPIM. Follicular antrum and theca interna layers are visible, allowing individual follicles to be identified easily. Follicles of all sizes can be seen and additional morphological details like Atretic follicles, blood vessels, interstitial connective tissue layer between adjacent follicles could also be seen. Out of all the visible morphological features, the SPIM images were analyzed for the most clinically or diagnostically relevant ones namely the size and number of follicles, the size of the developing COC inside the follicle and the follicular wall thickness.

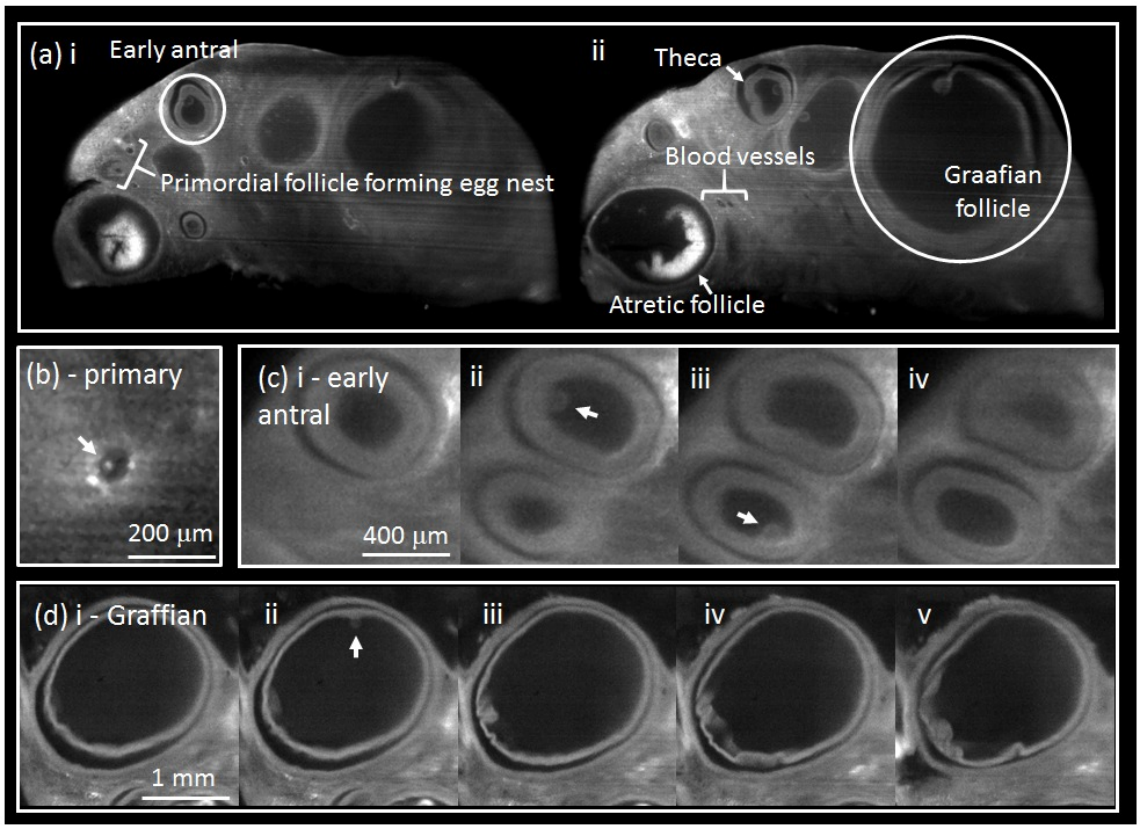

Figure 3. SPIM imaging results of ovarian follicles (a) Full FOV cross-sectional SPIM images taken at two different planes of a small follicle cluster. A few features are indicated: (i) early antral follicle and primordial follicle forming egg nest; (ii) Graafian and atretic follicles, theca layer, and blood vessels. (b) Primary follicle - diameter: 73 µm, COC: 29 µm. (c) Early antral follicles – diameter 586 µm (top) and 476 µm (bottom). Errors indicate COCs, which were measured to be 101 µm (top) and 98 µm (bottom). (d) Graafian follicle - diameter: 1788 µm, COC: 104 µm.

One complexity in the measurements of follicle sizes arose from their irregular shapes, therefore two orthogonal measurements intersecting at the midpoint of the first measurement were always taken for each follicle. The average of these two measurements was then used as the final follicle diameter value. All analysis were done manually. The dimensions of several follicles and their Cumulus Oophorus Complex (COC) were measured. According to the physical dimensions, the analyzed follicles can be categorized into the four main developmental stages of primordial, primary, antral and Graafian follicles. The detected follicle sizes varied from 70 µm up to 2.5 mm. A cluster of Primordial follicles, forming the egg nest, was detected as seen in fig. 3a. Typically in human at 18 – 22 weeks post-conception, the cortex of the ovary contains about 4 to 5 million primordial follicle. In Fig. 3b, a primary follicle was measured to have a diameter of 73 µm, with a primary oocyte. The Primary follicles develop receptors to follicle stimulating hormone (FSH) and oocyte genome is activated and genes become transcribed. Antral follicles typically measure between 100 µm (early antral) and 700 µm. Fig. 3c shows two early antral follicles: 590 µm (top) and 480 µm (bottom). These follicles show a clear antral cavity surrounded by a smooth theca interna layer clearly defining the follicle boundaries. COCs can be seen in panel (ii) and (iii) respectively. Fig. 3d shows a 1.8 mm Graafian follicle, and on panel (ii) the COC attached to the inner wall of the follicle can be clearly seen. In the present study, COC were detected in approximately 70 % of the follicles and the size of the COC vary from 40 µm - 110 µm. The lowest COC size detected was 40 µm which is comparable to histological finding and thus is a manifold improvement over magnification offered by ultrasound bio microscopy.

The relationship of oocyte sizes verses follicle sizes are shown in Fig. 4a. The measurements taken from SPIM data points are plotted as blue circles. To validate these quantitative observations, histological data of porcine ovarian follicles previously reported in literature [10] are also shown (red) in the same graph. The adjusted coefficient of determination (R-squared) between the SPIM data and the literature fitted curve was found to be 0.76, indicating that they are in good agreement. This result validates the SPIM technique as a quantitative method capable of follicle characterization.

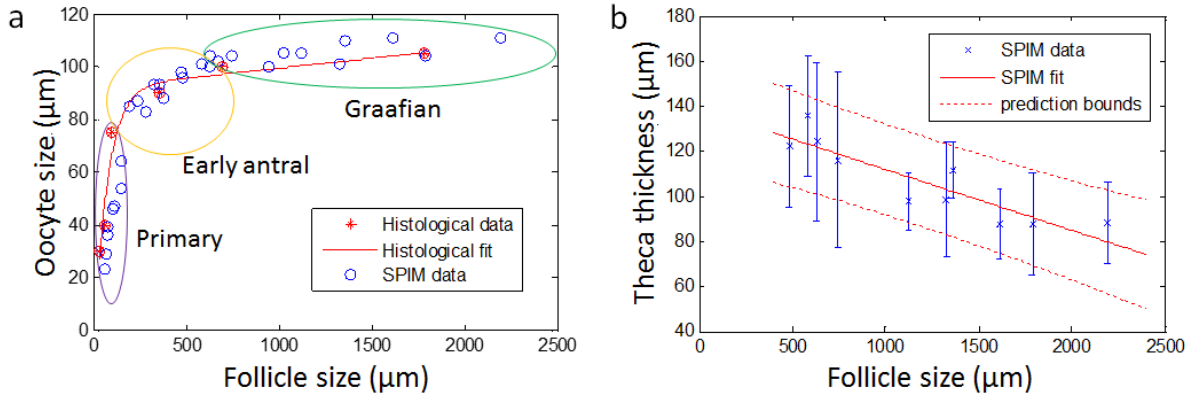

Figure 4. (a) Oocyte size versus follicle size. Blue circles are data points taken from the SPIM images (N = 30). Histological data of porcine ovarian follicles previously reported in literature are shown in red. R2 (adjusted coefficient of determination) = 0.76, indicating good agreement. Follicles can be categorised according to physical dimensions as: primary (violet) with COC ~60µm, early antral (yellow), with COC ~80µm, or Graafian follicles (green) with COC ~120µm. (b) Theca wall thickness versus follicle size measured from the SPIM data (N = 10). A maximum and minimum were taken for each follicle, and the average calculated. A linear fit was performed on the averaged values, along with the prediction bounds.

A very important feature of the SPIM follicle images was the distinctive visualization of the follicular thecal walls. The wall of the follicles comprises hormone-producing cells that are responsible for the production of fluid that is contained by the follicle wall. The theca wall thicknesses were measured from early antral and Graafian follicles. Fig. 4b shows these measurements distribution for different follicle sizes. It should be noted that the wall thickness is not uniform in the follicles. Hence, a maximum and minimum were taken from the SPIM images, and a linear fit was performed on the averaged values. In the study, a clear correlation was seen between the size of the follicle and the thecal wall thickness.

For the largest sized Graafian follicle the measured wall thicknesses were around 90 μm, which is lower than the thicknesses of antral follicle walls measured at around 120 μm. It is well established that identification of the follicle wall is useful in the characterization of different classes of developing follicles because the walls of follicles that are destined to ovulate are thinner [3].

A comparable method used currently to monitor ovarian follicles is ultrasonic biomicroscopy. As it is a non-invasive method the tissue remains undamaged after imaging, and thus offers the advantage of in-vivo imaging. Despite this, the low contrast and poor resolution of ultrasound imaging produces an image quality which is inferior to that of SPIM. Ultrasound fails to detect the primordial or primary follicles in the ovary and it seldom can detect growing COC in the Graafian follicle [11]. Although SPIM cannot be offered as a tool for in-vivo biological studies, its superior image quality offers valuable morphological details which may be of great help in developmental studies. For this particular case of ovarian follicle imaging, the smallest anatomical feature is >10 μm. The lateral resolution of 6 μm offered by the SPIM system allowed the imaging of features relevant and meaningful to the biological study. The current standard imaging method used for anatomical ovarian investigations is conventional H&E staining. While it provides the most detailed ex vivo information of all techniques due to its unparalleled resolution, it is not favor this application as the extra resolution would not reveal additional useful information. On the other hand, SPIM does offer advantages over histology in experimental practice. In SPIM imaging, as no physical slicing is required, excessive tissue processing is avoided. Not only does this preserve the valuable morphological information intact, the process is also time and labor efficient. Data acquisition is also a fast and automated procedure. The large FOV enables full coverage of any follicle clusters of up to 10 mm in its entirety. The full diameter within the sample is imaged in a single 2D image acquisition, and when combined with a high-speed camera and a motorized sample translation stage, a short imaging time of a few minutes can be achieved.

4. CONCLUSION

The novel combination of clearing and imaging of porcine ovarian follicles with SPIM was performed. The follicular antrum and theca interna layers were distinctly visible, allowing individual follicles to be easily identified. Follicles of all developmental stages were identified and their diameters characterized, ranging from the small primordial follicles up to 2.5 mm large Graafian follicles. Clearly distinguishable COCs protruding into the follicular antrum of several antral and Graafian follicles were measured at 40 μm - 110 μm in size, and their correlation with the developmental stage of the follicles were found to be in agreement with the literature. Measurements of theca thickness for follicles of different developmental stages were conducted, yielding results ranging from 90 μm to 120 μm. The good resolution and high contrast offered by SPIM images provides more quantitative anatomical information than ultrasound biomicroscopy images. The capability of the SPIM system to image a 3D follicle without destroying its morphological integrity coupled with the nature of high speed and low labor needs makes it also more preferable than the conventionally used H&E. SPIM is a suitable tool for ovarian follicle developmental stages characterization, without the need to extract oocytes from their natural environments. Any abnormalities in the physical dimensions could also be indicative of possible ovarian diseases, and these precise folliculometric measurements can be of immense help in improving the understanding of ovarian physiology, as well as help defining newer application areas including proper management of ovarian diseases.

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