

# Journal of Biomedical Optics

[SPIEDigitalLibrary.org/jbo](http://SPIEDigitalLibrary.org/jbo)

## **Rapid and nondestructive method for evaluation of embryo culture media using drop coating deposition Raman spectroscopy**

Zufang Huang  
Yan Sun  
Jing Wang  
Shengrong Du  
Yongzeng Li  
Juqiang Lin  
Shangyuan Feng  
Jinping Lei  
Hongxin Lin  
Rong Chen  
Haishan Zeng

# Rapid and nondestructive method for evaluation of embryo culture media using drop coating deposition Raman spectroscopy

Zufang Huang,<sup>a\*</sup> Yan Sun,<sup>b\*</sup> Jing Wang,<sup>a</sup> Shengrong Du,<sup>b</sup> Yongzeng Li,<sup>a</sup> Juqiang Lin,<sup>a</sup> Shangyuan Feng,<sup>a</sup> Jinping Lei,<sup>a</sup> Hongxin Lin,<sup>a</sup> Rong Chen,<sup>a</sup> and Haishan Zeng<sup>a,c</sup>

<sup>a</sup>Fujian Normal University, Key Laboratory of Opto-Electronic Science and Technology for Medicine of Ministry of Education, Fujian Provincial Key Laboratory for Photonics Technology, Fuzhou 350007, China

<sup>b</sup>Fujian Maternal and Child Health Hospital, Fuzhou 350001, China

<sup>c</sup>Imaging Unit—Integrative Oncology Department, British Columbia Cancer Agency Research Centre, Vancouver, British Columbia V5Z 1L3, Canada

**Abstract.** In this study, a rapid and simple method which combines drop coating deposition and Raman spectroscopy (DCDR) was developed to characterize the dry embryo culture media (ECM) droplet. We demonstrated that Raman spectra obtained from the droplet edge presented useful and characteristic signatures for protein and amino acids assessment. Using a different analytical method, scanning electron microscopy coupled with energy dispersive X-ray analysis, we further confirmed that Na, K, and Cl were mainly detected in the central area of the dry ECM droplet while sulphur, an indicative of the presence of macromolecules such as proteins, was mainly found at the periphery of the droplet. In addition, to reduce sample preparation time, different temperatures for drying the droplets were tested. The results showed that drying temperature at 50°C can effectively reduce the sample preparation time to 6 min (as compared to 50 min for drying at room temperature, ~25°C) without inducing thermal damage to the proteins. This work demonstrated that DCDR has potential for rapid and reliable metabolomic profiling of ECM in clinical applications. © The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: 10.1117/1.JBO.18.12.127003]

Keywords: Raman spectroscopy; embryo culture media; drop coating deposition; scanning electron microscopy; energy dispersive x-ray analysis.

Paper 130627TNR received Aug. 28, 2013; revised manuscript received Oct. 31, 2013; accepted for publication Oct. 31, 2013; published online Dec. 16, 2013.

## 1 Introduction

Assessment of oocyte embryo quality helps identify the embryo with highest likelihood of implantation and the greatest pregnancy potential. Usually, the embryos selected for transfer are chosen according to morphological criteria and rate of development in culture on microscopic assessment, which is considered to be simple and practical over other methods for scoring the oocytes and embryos. Although morphological evaluation is noninvasive, the problem is that relying solely on morphology assessment may not accurately predict the ability of an embryo to implant<sup>1</sup>. In addition, morphology assessment always requires skilled personnel and is difficult to standardize. Therefore, methods that can provide a reliable and objective assessment of a given embryo are highly desired. The metabolism of an embryo requires the uptake of certain substances from the surrounding culture media, and the changes of metabolites' concentrations in culture media may reflect cellular activities and overall developmental potential during the culture period. Recent studies have suggested that metabolomic profiling of embryo culture media (ECM) can identify human embryos

with better implantation potential.<sup>2-3</sup> Currently, investigations of the complex metabolic/metabolomic profiles of biological systems are mainly performed by nuclear magnetic resonance spectroscopy and liquid chromatography-mass spectrometry (LC-MS).<sup>3-5</sup> However, the complexity of operation and the high costs of these methods have impeded their wide applications in clinical settings. By contrast, Raman spectroscopy, which detects vibrations in molecules, allows one to characterize biological samples by providing unique spectral patterns originating from their inherent biochemical compositions. In addition, the major advantages of Raman spectroscopy are ease of use, immediate results, cost-effective, and label-free analysis, making it an increasingly popular tool for probing the biological samples.

So far, a handful of studies have been conducted on metabolomic profiling of ECM with the use of Raman spectroscopy. Seli et al.<sup>6</sup> first reported the use of Raman spectroscopy for analysis of ECM to predict pregnancy outcomes. Recently, Shen et al.<sup>7</sup> have achieved an 85.7% diagnostic accuracy with clinical pregnancy using Raman spectroscopy under the guidance of morphological assessment by analyzing the relative fitting coefficients of phenylalanine/albumin and pyruvate/albumin. However, in these studies, a relatively long spectral acquisition time is needed (~5 min) in order to achieve high quality Raman spectra in ECM solutions. Variations in probing volume during long period of measurement time are unavoidable, which may lead to the lack of reproducibility. Therefore, it is desirable to develop new methods for rapid and reliable evaluation.

\*These authors contributed equally to this work.

Address all correspondence to: Rong Chen, Fujian Normal University, Key Laboratory of Opto-Electronic Science and Technology for Medicine of Ministry of Education, Fujian Provincial Key Laboratory for Photonics Technology, Fuzhou 350007, China. Tel: +86 591 83489919; Fax: +86 591 83465373; E-mail: [chenr@fjnu.edu.cn](mailto:chenr@fjnu.edu.cn) or Haishan Zeng, Imaging Unit—Integrative Oncology Department, British Columbia Cancer Agency Research Centre, Vancouver, BC V5Z 1L3, Canada. E-mail: [hzeng@bccrc.ca](mailto:hzeng@bccrc.ca)

Drop deposition, known as the “coffee ring” effect, is a very simple technique, in which a fluid droplet dries on a solid, flat substrate.<sup>8</sup> And this coffee ring pattern can result in significant constituent preconcentration.<sup>9</sup> Recently, Raman spectroscopy has been combined with drop deposition, also known as drop coating deposition Raman (DCDR), to study protein mixtures and biofluids.<sup>9–11</sup> Relevant results demonstrate that this technique can facilitate the segregation of different chemicals or biopolymers to achieve preconcentration, thus providing highly reproducible Raman spectra with high signal-to-noise ratio.

Here, to the best of our knowledge, we demonstrate for the first time, the application of DCDR for the rapid and sensitive detection of ECM. In addition, the spatial distribution of biochemical compositions in the dried ECM droplet was also evaluated.

## 2 Materials and Methods

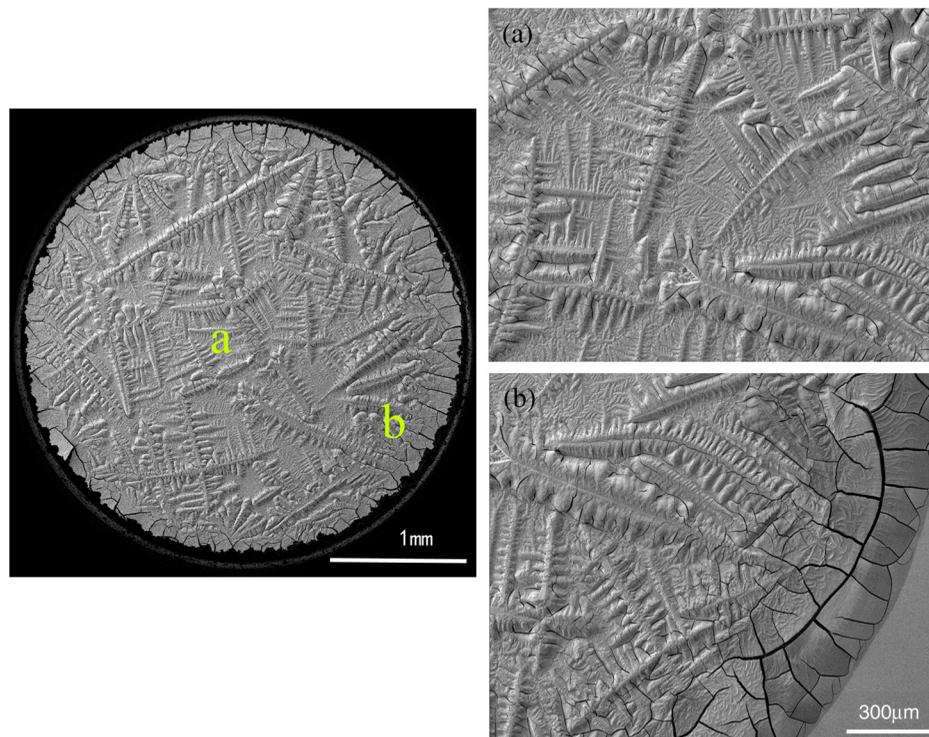
Embryo culture media (IVF-30 was purchased from Vitrolife), (Sweden) and was used without further purification. Volume of 10  $\mu$ l ECM was aspirated with a micropipette and then was directly spotted onto pre-cleaned (rinsed twice by ultra-purified water before usage) stainless steel substrate (Z&S Tech, Starkville) For DCDR preparation, an aliquot of ECM was passively dried at room temperature for nearly 50 min, and another set of ECM solution were aliquoted and dried under different temperatures by using the drying oven (DHG-9140, Yiheng Co., Shanghai, China).

Raman spectra were recorded by a micro-Raman spectroscopy system (Renishaw Invia, UK) using a 785-nm laser excitation with  $\sim$ 92 mW of power. A 20 $\times$  objective lens (NA = 0.4, Leica, Germany) with a spot size of 2.5  $\mu$ m  $\times$  50  $\mu$ m was used to focus the excitation beam and collect the backscattered Raman

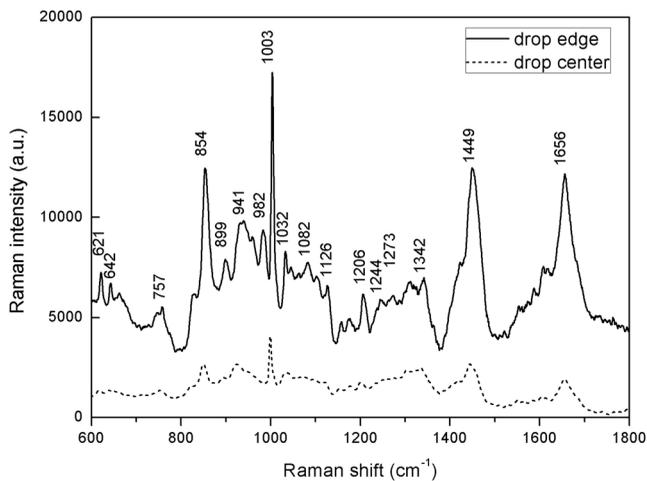
signals from samples in standard confocal mode. A 1200 line/mm grating was used to scan a spectral range of 600 to 1800  $\text{cm}^{-1}$ . Raman signal detection was carried out by a Peltier cooled charge-coupled device (CCD) camera with an integration time of 30 s. Three replicates were taken per droplet, which means one spectrum at a time. After obtaining the ECM spectra, Savitsky-Golay smoothing method (5th polynomial order) in WiRE 2.0 software programme was used to smooth the Raman data, and the “curve fit” function was used to extract the peak intensity. Prior to Raman experiment, calibration was performed with reference to the 520- $\text{cm}^{-1}$  peak of silicon. In order to evaluate the spatial location of the ECM chemical components, scanning electron microscopy (SEM) images were obtained on a JSM-7500F field emission scanning electron microanalyzer (JEOL, Japan) coupled with an energy dispersive x-ray spectrometer.

## 3 Results and Discussion

Figure 1 shows a typical low magnification SEM micrograph image of a dried ECM droplet. The heterogeneous deposit formed two main regions: a cracked ring along the edge and a fern-shaped precipitate in the center. Explanation of the droplet (including proteins and other analytes) drying process has been well characterized.<sup>12</sup> With the evaporating of water, the protein is deposited at the droplet margin and the concentrations of salts continue to increase. When the solubility limit of the inorganic salt is reached, spontaneous fern-like precipitate formation occurs. Previous studies have shown that the fern dendrites were mainly made up of NaCl and KCl.<sup>12–14</sup> Cracked thin film due to the dehydration observed at the edge of the dried ECM droplet contains the typical less soluble proteins. Figure 2 presents Raman spectra obtained from the dried ECM



**Fig. 1** Micrograph image of dried embryo culture media (ECM) drop under scanning electron microscopy, and their corresponding enlarged images: (a) center region and (b) periphery area.

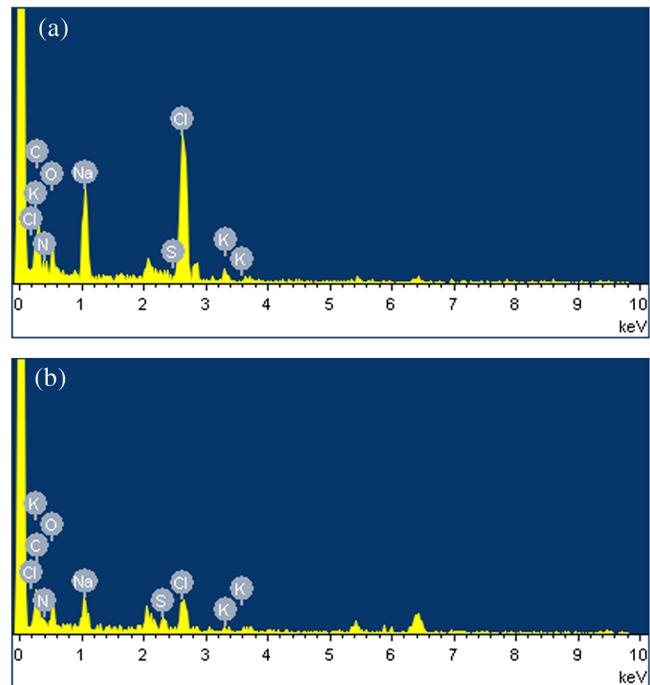


**Fig. 2** Raman spectra (background corrected) obtained from ECM droplet edge and center areas in the 600 to 1800  $\text{cm}^{-1}$  region. Spectra are vertically shifted for clarity.

droplet; the lower curve (dash line) indicates the Raman spectrum obtained from the center region. As can be seen, the signal-to-noise ratio is relatively poor, and this is mainly due to the fact that the ferns mainly contain Raman inactive inorganic salts, such as NaCl, KCl, and a small amount of proteins.<sup>15</sup> By contrast, the upper curve gives a Raman spectrum with high signal-to-noise ratio collected from the droplet edge. Most bands assigned to vibrational modes of biomolecules, such

**Table 1** Peak positions and tentative assignments of major vibrational bands observed in ECM.<sup>7,19</sup>

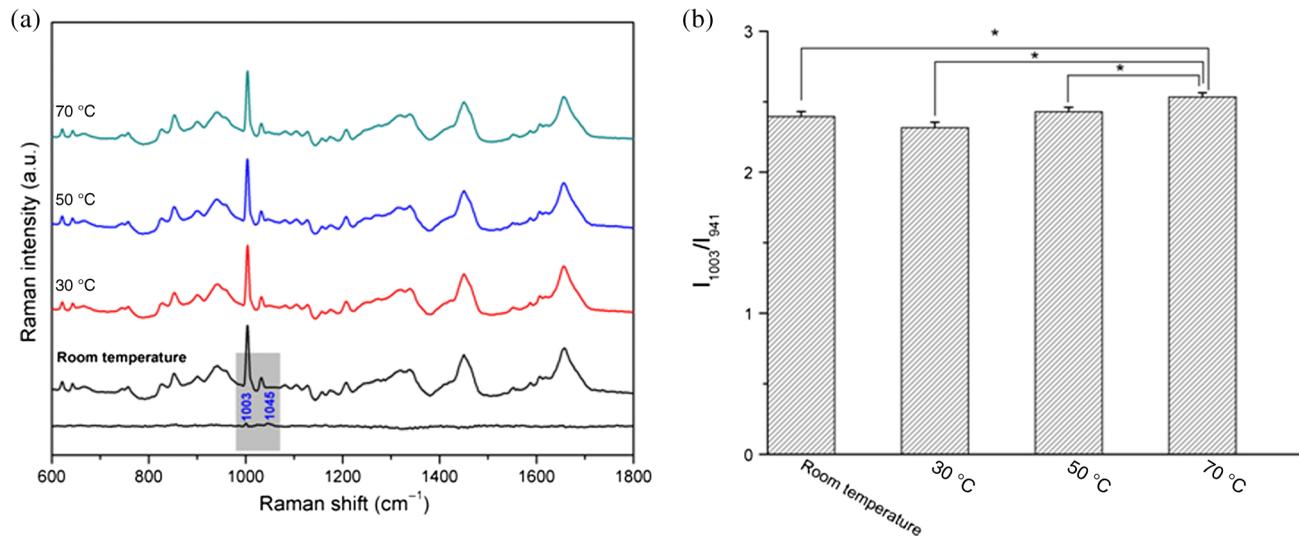
Raman shift ( $\text{cm}^{-1}$ )	Assignments
621	C–C twisting mode of phenylalanine
642	C–C, wagging tyrosine
757	tryptophan
854	tyrosine and proline
899	C <sub>1</sub> –H, vibrational mode of protein
941	C–C skeleton stretching mode of protein, proline
982	tryptophan
1003	Phenylalanine, $\nu_s$ symmetric ring breathing mode
1032	C–H bending mode of phenylalanine
1082	C–C or C–O stretching mode, lipids; $\text{PO}_2$ skeleton
1126	C–N stretching mode, proteins
1206	Hydroxyproline, tyrosine
1342	$\text{CH}_3\text{CH}_2$ wagging mode of collagen
1449	$\text{CH}_2$ bending mode, proteins
1656	Amide I (C=O stretching mode of proteins, $\alpha$ -helix conformation)



**Fig. 3** Energy dispersive x-ray analysis of the fern-shaped region (a) and cracks at the droplet edge (b) shown in Fig. 1.

as proteins and amino acids were clearly visible, e.g., phenylalanine ring breathing band  $1003 \text{ cm}^{-1}$ , tyrosine, proline band at  $854 \text{ cm}^{-1}$ ,  $\text{CH}_2$  bending mode at  $1449 \text{ cm}^{-1}$ , and Amide I band at  $1656 \text{ cm}^{-1}$ . Detailed tentative band assignments are shown in Table 1. By careful comparison, we found that the Raman spectra obtained from the droplet edge agrees quite well with the previous result given by Shen et al. obtained from the culture media.<sup>7</sup> Our spectral acquisition time is ten times shorter than in their experiment. Previous study has shown that protein and buffer species can segregate well upon deposition, especially for simple mixtures with relatively low concentrations.<sup>11,16</sup> However, compounds with a strong chemical affinity for each other are not expected to easily segregate.<sup>17</sup> A similar phenomenon has been observed in Raman spectra obtained from a dried synovial fluid droplet. One possible explanation is that the more soluble and the light-weight protein species may precipitate in the droplet center.<sup>18</sup> This suggests that spectra collected from the droplet edge were composed primarily of protein and amino acids macromolecules Raman bands. This may provide sufficient data for evaluating the physiochemical composition of ECM, although the coarse separation does not prevent the proteins precipitating in the center regions. The EDXA shows that fern-patterns in the center regions were mainly composed of sodium and chloride [Fig. 3(a)], while in the droplet edge, in addition to sodium and chlorine, it showed a much more intensive peak from sulphur [Fig. 3(b)], indicating the aggregation of proteins.

Previous studies have shown that, generally, microliters of droplets were needed to dry at room temperature for a long time ranging from tens of minutes and up to several hours before Raman spectral measurement.<sup>20–21</sup> Here, we demonstrate a simple and rapid method to speed up evaporation to achieve fast drying by increasing the sample temperature (see Sec. 2). Compared with the long time (50 min) needed for passive air-dry at room temperature, the drying process takes a much



**Fig. 4** (a) Comparison of Raman spectra obtained from droplet edge under different drying temperatures. Raman spectra are offset for clarity. Also shown at the bottom is the difference spectrum (50°C minus room temperature). (b) Histograms displaying the average intensity ratio of 1003 to 941 cm<sup>-1</sup>, under different drying temperature. \* indicates a significant difference ( $P < 0.05$ ).

shorter time to prepare the ECM samples (~12 min for 30°C, ~6 min for 50°C, and ~2 min for 70°C). As shown in Fig. 4(a), Raman spectra from the droplet edge of the same sample under different drying temperatures (room temperature, 30°C, 50°C, and 70°C), demonstrate almost the same Raman spectral profile (e.g., the related coefficients between spectra obtained at room temperature and 50°C is 0.996) and the shaded area indicates the region where only two minor differences at Raman peaks of 1003 and 1045 cm<sup>-1</sup> were found. It should be noted that the latter was presented as a shoulder band, which is not always reliably detected. One potential concern of increasing the drying temperature is causing thermal-induced to the proteins. Previous study has reported that intensity of the 946-cm<sup>-1</sup> band is associated with the content of proteins in the  $\alpha$ -helical state, and upon heating, the  $\alpha$ -helical conformation will become degraded and the increase in the  $I_{1004}/I_{946}$  ratio suggests a vital change in protein conformation.<sup>22</sup> Our calculated  $I_{1003}/I_{941}$  intensity ratios in these four groups (room temperature, 30°C, 50°C, and 70°C) were shown in Fig. 4(b). The intensity ratio (as mentioned) between different drying temperatures was compared and the significance of the differences  $P < 0.05$  was analyzed using Student t test in the SPSS 15.0 software package (SPSS Inc., Chicago). It can be seen that although intensity ratios were similar, after careful comparison, the intensity ratios of 1003 to 941 cm<sup>-1</sup> between 70°C and other three groups indicated significant difference ( $P < 0.05$ ), suggesting a drying temperature of 70°C does bring somewhat thermally induced denature to protein. However, there is no significant difference ( $P > 0.05$ ) among the other three groups. Low temperature implies a slow evaporation rate and eventually a long time needed to prepare samples, a drying temperature of 50°C would be optimal for rapid dried ECM sample preparation, and consequently to achieve the stable and satisfactory Raman data. Pending further validation, the value derived in this study should not be considered a universal value to be applied to all samples by all instruments.

In principle, parameters such as fluid properties, solute-substrate interaction, intermolecular forces, and drying conditions

were reported<sup>23–26</sup> to affect droplet shapes and the spatial distribution of analytes (proteins and salts) by showing different deposition patterns. Although Raman spectra obtained from a dried droplet edge would be information rich and reliable for further characterization and comparison between ECM samples at different stages during embryo development, morphological evaluation of embryo coupled with the corresponding ECM Raman spectra would be optimal for predicting embryo developmental and implantation potential. It should be noted that the higher drying temperatures used can speed up the drying time, however it does raise the possibility to not only denature but also dehydrate the proteins. Other strategies, such as applying a slight vacuum to the deposited samples at room temperatures may also significantly decrease the drying time. Therefore one essential prerequisite is that uniform and carefully controlled conditions should be guaranteed during the sample preparation.<sup>27</sup>

## 4 Conclusion

In summary, DCDR demonstrates a simple, sensitive, and highly effective way for analyzing ECM. This nondestructive analysis would also allow the same biofluid droplet to be easily examined with other techniques. In addition, in a proper range of increasing the drying temperature, one can significantly reduce the sample preparation time and keep the sample from being thermally damaged. We believe that the DCDR technique has the potential of being applied to noninvasively assess embryo quality by analyzing metabolomic profiling of ECM.

## Acknowledgments

This project was supported by the National Natural Science Foundation of China (grant Nos. 61308113, 61178090, 81101110, and 11274065), the Key Clinical Specialty Discipline Construction Program of Fujian, P.R.C(20121589), the Natural Science Foundation of Fujian Province, China (No. 2013J01225), and the Program for Changjiang Scholars and Innovative Research Team in University (IRT1115).

## References

1. A. A. Milki et al., "Comparison of blastocyst transfer with day 3 embryo transfer in similar patient populations," *Fertil. Steril.* **73**(1), 126–129 (2000).
2. R. Scott et al., "Noninvasive metabolomic profiling of human embryo culture media using Raman spectroscopy predicts embryonic reproductive potential: a prospective blinded pilot study," *Fertil. Steril.* **90**(1), 77–83 (2008).
3. Z. P. Nagy et al., "Metabolomic assessment of oocyte viability," *Reprod. Biomed. Online* **18**(2), 219–225 (2009).
4. L. Botros, D. Sakkas, and E. Seli, "Metabolomics and its application for non-invasive embryo assessment in IVF," *Mol. Hum. Reprod.* **14**(12), 679–690 (2009).
5. E. Seli et al., "Noninvasive metabolomic profiling of embryo culture media using proton nuclear magnetic resonance correlates with reproductive potential of embryos in women undergoing in vitro fertilization," *Fertil. Steril.* **90**(6), 2183–2189 (2008).
6. E. Seli et al., "Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing in vitro fertilization," *Fertil. Steril.* **88**(5), 1350–1357 (2007).
7. A. G. Shen et al., "Accurate and noninvasive embryos screened during in vitro fertilization (IVF) assisted by Raman analysis of embryos culture medium," *Laser Phys. Lett.* **9**(4), 322–328 (2012).
8. R. D. Deegan et al., "Capillary flow as the cause of ring stains from dried liquid drops," *Nature* **389**(6653), 827–828 (1997).
9. I. Barman et al., "Raman spectroscopy-based sensitive and specific detection of glycosylated hemoglobin," *Anal. Chem.* **84**(5), 2474–2482 (2012).
10. J. Filik and N. Stone, "Drop coating deposition Raman spectroscopy of protein mixtures," *Analyst* **132**(6), 544–550 (2007).
11. D. Zhang et al., "Raman detection of proteomic analytes," *Anal. Chem.* **75**(21), 5703–5709 (2003).
12. I. H. Segel, *Biochemical Calculations*, Wiley New York (1976).
13. E. I. Pearce and A. Tomlinson, "Spatial location studies on the chemical composition of human tear ferns," *Ophthalmic Physiol. Opt.* **20**(4), 306–313 (2000).
14. F. Chrétien and J. Berthou, "A new crystallographic approach to fern-like microstructures in human ovulatory cervical mucus," *Hum. Reprod.* **4**(4), 359–368 (1989).
15. J. Filik and N. Stone, "Analysis of human tear fluid by Raman spectroscopy," *Anal. Chim. Acta* **616**(2), 177–184 (2008).
16. C. Ortiz et al., "Validation of the drop coating deposition Raman method for protein analysis," *Anal. Biochem.* **353**(2), 157–166 (2006).
17. D. Zhang et al., "Chemical segregation and reduction of Raman background interference using drop coating deposition," *Appl. Spectrosc.* **58**(8), 929–933 (2004).
18. K. A. Esmonde-White et al., "Raman spectroscopy of dried synovial fluid droplets as a rapid diagnostic for knee joint damage," *Proc. SPIE* **68539**, 68530Y (2008).
19. A. Synytsya et al., "Raman spectroscopic study of serum albumins: an effect of proton- and  $\gamma$ -irradiation," *J. Raman Spectrosc.* **38**(12), 1646–1655 (2007).
20. K. A. Esmonde-White et al., "Raman spectroscopy of synovial fluid as a tool for diagnosing osteoarthritis," *J. Biomed. Opt.* **14**(3), 034013 (2009).
21. E. Kočíšová and M. Procházka, "Drop-coating deposition Raman spectroscopy of liposomes," *J. Raman Spectrosc.* **42**, 1606–1610 (2011).
22. C. Xie et al., "Study of dynamical process of heat denaturation in optically trapped single microorganisms by near-infrared Raman spectroscopy," *J. Appl. Phys.* **94**(9), 6138–6142 (2003).
23. E. Adachi, A. S. Dimitrov, and K. Nagayama, "Stripe patterns formed on a glass surface during droplet evaporation," *Langmuir* **11**(4), 1057–1060 (1995).
24. H. Hu and R. G. Larson, "Marangoni effect reverses coffee-ring depositions," *J. Phys. Chem. B* **110**(14), 7090–7094 (2006).
25. A. P. Sommer, "Microtornadoes under a nanocrystalline igloo: results predicting a worldwide intensification of tornadoes," *Cryst. Growth Des.* **7**(6), 1031–1034 (2007).
26. A. P. Sommer and N. Rozlosnik, "Formation of crystalline ring patterns on extremely hydrophobic supersmooth substrates: extension of ring formation paradigms," *Cryst. Growth Des.* **5**(2), 551–557 (2005).
27. J. V. Kopecký and V. Baumruk, "Structure of the ring in drop coating deposited proteins and its implication for Raman spectroscopy of biomolecules," *Vib. Spectrosc.* **42**, 184–187 (2006).