

# Combining Optical Quadrature and Differential Interference Contrast to facilitate embryonic cell counting with Fluorescence Imaging for confirmation

William C. Warger II\*<sup>1</sup>, Judith A. Newmark<sup>2</sup>, ChihChing Chang<sup>1</sup>, Dana H. Brooks<sup>1</sup>,  
Carol M. Warner<sup>2</sup>, Charles A. DiMarzio<sup>1</sup>

<sup>1</sup>Northeastern University, 440 Dana, 360 Huntington Ave., Boston, MA 02115

<sup>2</sup>Northeastern University, 134 Mugar Building, 360 Huntington Ave., Boston, MA 02115

## ABSTRACT

The Multifunctional Staring Mode Microscope was developed to permit three modes of imaging for cell counting in mouse embryos: Optical Quadrature, Differential Interference Contrast (DIC), and Fluorescence Imaging. The Optical Quadrature Microscope, consisting of a modified Mach-Zender Interferometer, uses a 632.8 nm laser to measure the amplitude and phase of the signal beam that travels through the embryo. Four cameras, preceded by multiple beamsplitters, are used to read the four interferograms, which are then combined to produce an image of the complex electric field amplitude. The phase of the complex amplitude is then unwrapped using a 2-D phase unwrap algorithm and images of optical path length are produced. To combine the additional modes of DIC and Fluorescence Imaging with the Optical Quadrature Microscope, a 632.8 nm narrow bandpass beamsplitter was placed at the output of the microscope. This allows the laser light to continue through the Mach-Zender while all other wavelengths are reflected at 90 degrees to another camera. This was effective in combining the three modes as the fluorescence wavelength for the Hoechst stain is well below the bandpass window of the beamsplitter. Both live and fixed samples have been successfully imaged in all three modes. Accuracy in cell counting was achieved by using the DIC image for detecting cell boundaries and the Optical Quadrature image for phase mapping to determine where cells overlap. The final results were verified by Hoechst fluorescence imaging to count the individual nuclei. Algorithms are currently being refined so larger cell counts can be done more efficiently.

Keywords: microscopy, embryo, cell counting, Optical Quadrature, Differential Interference Contrast, multi-modal

## 1. INTRODUCTION

Over one million babies have been born by *in vitro* fertilization (IVF) since the birth of the first IVF baby in 1978<sup>1</sup>. In order to increase the success rate of IVF, doctors transfer multiple embryos back into the mother. The transfer of three embryos has long been the standard; however there is a slow change toward reducing the standard because studies have shown that the transfer of two embryos does not decrease the overall ongoing pregnancy rate. This method has eliminated most triplets but does not reduce the rate of twin pregnancies<sup>2-8</sup>. However, even with the transfer of two embryos many IVF clinics report an overall pregnancy rate of only 30-40%<sup>9</sup>, with 20-40% of these successful births resulting in multiple pregnancies<sup>9-12</sup>. Between 1980 and 1997, the United States twin birth rate increased 42% and the triplet and higher order rate increased 500%<sup>13</sup>. The triplet rate has since decreased with the transfer of two embryos shown by 82% of procedures transferring three embryos in 1997 but only 67% of procedures in 2000<sup>13</sup>. However the rate of twin births has continued to rise, resulting in a multitude of problems for both mother and child, including pregnancy complications, preterm delivery, low birth weight, congenital malformations, and infant death<sup>14-20</sup>. Therefore, there is a worldwide thrust to identify one healthy embryo for transfer back to the mother<sup>22</sup>.

One reason for the low success rate of IVF is the uncertainty of whether an embryo is healthy enough to produce a successful pregnancy. To assess the quality of the embryo during the preimplantation period, the number of cells and the overall morphology of the embryo are recorded over the observation period<sup>10</sup>. One published set of assessment criteria states: four or five cells on day 2, seven or more cells on day 3, no more than 20% fragmentation, and the absence of

cells with multiple nuclei during the entire observation period<sup>21</sup>. However, it is believed that pregnancy rates are low for this method because the embryo would still be in the Fallopian tube during this stage of development in a natural pregnancy and not in the uterus where it is transferred during IVF<sup>23</sup>. Developments in embryo culture systems have allowed for viable embryos to develop *in vitro* into the blastocyst stage, consisting of more than 30 cells, on day 5 or 6<sup>23</sup>. With the extended observation time, better decisions can be made on the quality of the embryo and slightly better percentages of pregnancies have been recorded.

The number of cells during the one to eight cell stage is an important criterion for choosing a high quality embryo. However it is impossible to use this criterion past the eight cell stage due to the present imaging technology used by physicians. Centers presently use Differential Interference Contrast (DIC) microscopy to image the embryos non-invasively. This method shows distinct cell boundaries within the focal plane when multiple cells do not lie in the path to the microscope objective. Since an embryo is optically transparent, some cell edges can be seen when a couple of cells are in the path to the objective, allowing two layers of four cells to be imaged. For this reason accurate cell counts cannot be produced past the eight cell stage and therefore cannot be a decisive factor for a high quality embryo after the eight cell stage. Consequently, the criteria for a high quality embryo at the blastocyst stage are primarily based on the morphology of the sample and the rate of development. Thus, the creation of an instrument that can non-invasively count the number of cells during embryo development up to the blastocyst stage would help physicians make a better determination as to which one embryo to transfer, and may raise the success statistics of single embryo transfer and thus eliminate the motivation for multiple embryo transfers.

## 2. OPTICAL QUADRATURE MICROSCOPY

Optical Quadrature Microscopy uses a 632.8 nm laser within a modified Mach-Zender Interferometer setup to develop images of optical path difference. The circularly polarized reference beam ( $E_{ref}$ ) is mixed with the 45 degree linearly polarized signal beam ( $E_{sig}$ ) in a non-polarizing beamsplitter producing the outputs  $E_{sig} + E_{ref}$  and  $E_{sig} - E_{ref}$ , where ideally:

$$E_{ref} = E \cdot e^{j(\omega t + \phi)} (\mathbf{x} + j\mathbf{y}) \quad (1)$$

$$E_{sig} = A \cdot E \cdot e^{j(\omega t + \phi + \alpha)} (\mathbf{x} + \mathbf{y}). \quad (2)$$

Only one of these outputs is required to complete the quadrature equations, however it was found that using them both in a balanced-mixing configuration improved image quality<sup>24</sup>. The  $A \cdot e^{j\alpha}$  in Equation 2 is the magnitude and phase induced by the sample on the signal arm. Two polarizing beamsplitters (PBS) and four corresponding CCD cameras follow the recombining non-polarizing beamsplitter (NPBS), as seen in Figure 1, to capture the fields:

$$\text{Camera 0: } |E_{ref}|^2 + |E_{sig}|^2 + 2\text{Re}(E_{ref} \cdot E_{sig}^*) \quad (3)$$

$$\text{Camera 1: } |E_{ref}|^2 + |E_{sig}|^2 + 2\text{Im}(E_{ref} \cdot E_{sig}^*) \quad (4)$$

$$\text{Camera 2: } |E_{ref}|^2 + |E_{sig}|^2 - 2\text{Re}(E_{ref} \cdot E_{sig}^*) \quad (5)$$

$$\text{Camera 3: } |E_{ref}|^2 + |E_{sig}|^2 - 2\text{Im}(E_{ref} \cdot E_{sig}^*). \quad (6)$$

The four equations can be represented succinctly as:

$$M_n = |E_{ref}|^2 + |E_{sig}|^2 - 2\text{Re}(i^n \cdot E_{ref} \cdot E_{sig}^*). \quad (7)$$

By blocking the signal and reference arms individually and simultaneously we are able to capture images for the pure signal ( $S_n$ ), reference ( $R_n$ ), and detector dark voltage ( $D_n$ ). After subtraction of  $D_n$  from each of the components, the resultant image is reconstructed using:

$$E_r = \frac{1}{4} \sum_{n=0}^3 i^n \cdot \frac{M_n - S_n - R_n}{\sqrt{R_n}} \quad (8)$$

Fixed-pattern levels of the individual cameras caused by non-ideal components, including the beamsplitters and cameras, are not removed by common mode rejection or the subtraction of  $D_n$  because they are associated with the individual cameras and thus require the subtraction of  $S_n$  and  $R_n$ . The square root of  $R_n$  normalizes each component to ensure balanced intensities for balanced detection. Dividing the result of Equation 8 by a reconstructed blank image not containing a sample will give the magnitude and phase due to the sample:

$$A \cdot e^{j\alpha} = E_r / E_{r,blank} \quad (9)$$

Since phase has a range from zero to  $2\pi$  and the parameter of interest is the optical path length, equal to the integral of the index of refraction along the path, the phase image must be unwrapped using a 2D phase unwrapping algorithm. The  $L^p$ -norm algorithm<sup>25</sup> was experimentally found to work the best on a group of embryos tested. Then multiplying the phase intensities by  $2\pi / \lambda$ , where  $\lambda = 632.8 \text{ nm}$ , gives images of optical path length difference for the sample.

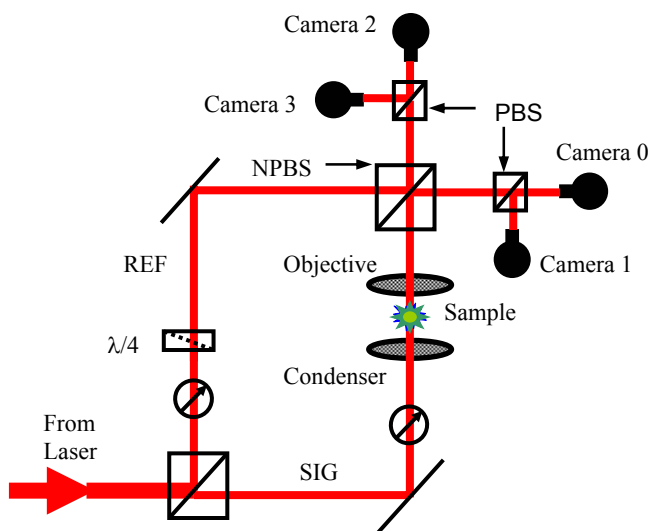


Figure 1: Layout for Optical Quadrature Microscopy

### 3. DIFFERENTIAL INTERFERENCE CONTRAST

DIC microscopy produces images from the gradient of optical path lengths along a given direction, in order to provide contrast for optically transparent samples. The white light source is sheared with a Wollaston prism into a reference and signal beam with a difference less than the diameter of an Airy disk. When these two beams are recombined with another Wollaston prism, the interference will result in high or low intensity depending on the increase or decrease in the difference of optical path length between the two paths. As the gradient of optical path length difference increases, the contrast within the image increases. The direction of the gradient is dependent upon the direction of polarization of the light source supplied by a linear polarizer before the first Wollaston prism.

### 4. EPI-FLUORESCENCE IMAGING

Fluorescence microscopy is the technique whereby a fluorescent substance is excited by a narrow range of wavelengths and the emitted, fluorescent, wavelengths are collected to form an image. Since the fluorescent light is emitted in all directions very low signals are captured within the numerical aperture of the objective, therefore restricting the number of optical elements in order to get a substantial signal-to-noise ratio at the detector. Although many materials are

naturally fluorescent, stains are used to bind fluorophores to specific substances within the sample. To image the nucleus of each cell we use Hoechst Stain which fluoresces in the blue when illuminated by UV light. Imaging the nuclei with will allow us to estimate the number of cells within the embryo. There is the possibility of multiple nuclei within a single cell after division of the nucleus but before full cell division. This method of cell counting would be ideal for physicians, however the Hoechst Stain permanently binds to the DNA of the nucleus and is therefore considered an invasive procedure.

### 5. MULTIFUNCTIONAL STARING MODE MICROSCOPE

A 620 – 650 nm narrow bandpass dichroic beamsplitter is positioned after the camera port of the Nikon ECLIPSE TE200, already configured for Optical Quadrature, to allow the 632.8 nm light to continue through to the recombining beamsplitter while reflecting the other visible wavelengths to an additional CCD camera, as seen in Figure 2. This allowed the additional CCD to capture images of DIC and Epi-Fluorescence, depending on the configuration of the microscope, with the least amount of Fresnel reflection from extra optical elements. A transform was created with a calibration target to register all images created by the Optical Quadrature CCDs to the new DIC/Fluorescence CCD. Both fixed, Figure 3, and live, Figure 4, embryos have been imaged and registered in all three modes.

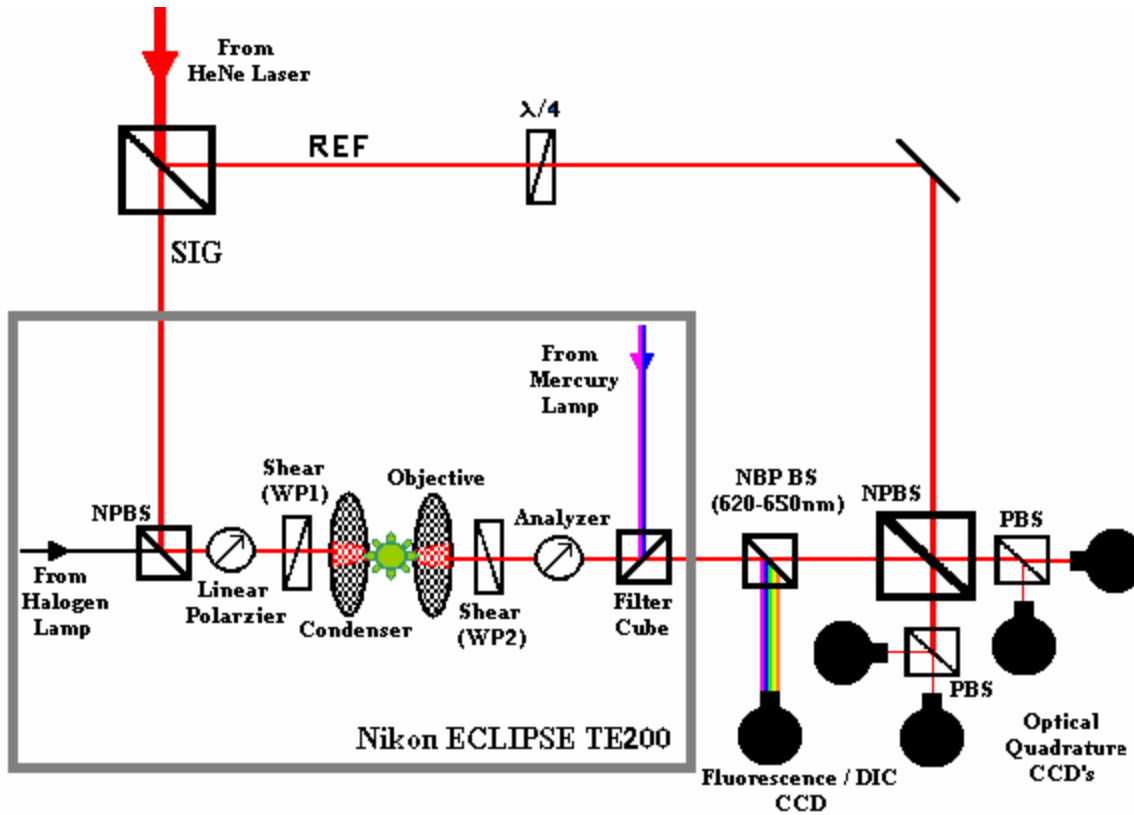


Figure 2: Layout for Multifunctional Staring Mode Microscope, that incorporates Optical Quadrature, DIC and Epi-Fluorescence into one multi-modal microscope.

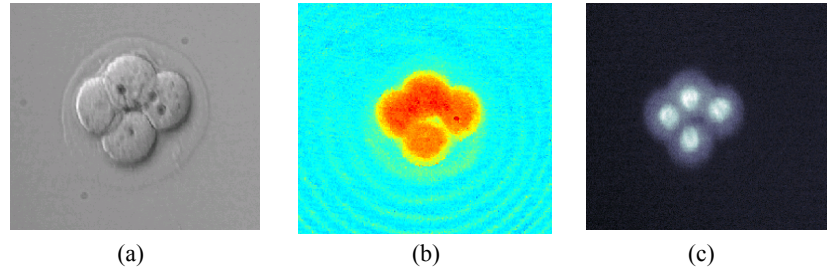


Figure 3: A four cell embryo imaged in (a) DIC, (b) Optical Quadrature, (c) Epi-Fluorescence of nuclei using Hoechst Stain

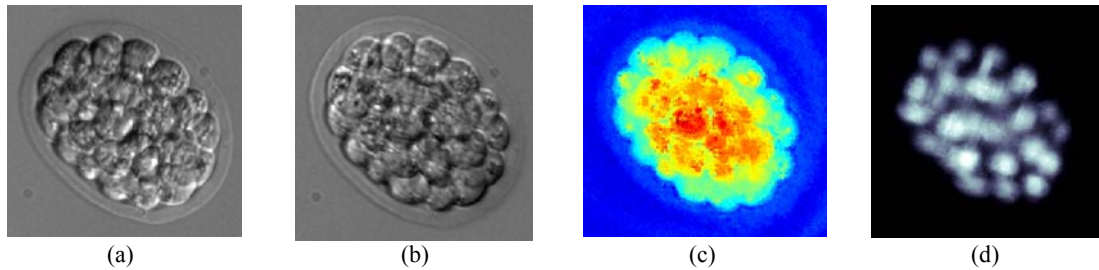


Figure 4: A twenty six cell embryo imaged in (a) DIC, (b) DIC with 90 degree polarization shift, (c) Optical Quadrature, and (d) Epi-Fluorescence of nuclei using Hoechst Stain.

## 7. PRELIMINARY CELL COUNTING RESULTS

We have developed a contour signature cell counting algorithm that only requires the Optical Quadrature image. The algorithm begins by using experimentally chosen threshold values to identify a distinct outer boundary of the embryo and applies Sobel edge filters to enhance it. A distance transform with Euclidean geometry is applied to the boundary to produce a separate gray level image to create a distinct shape for the sample, seen in Figure 5. The distance transform starts with an intensity value of zero at the boundary and increases until reaching a maximum value at the center of the object. This results in every point within the sample representing the distance to the nearest point on the boundary. A contour signature curve is created by recording the distance between the centroid and each point along the perimeter of the object. This curve is plotted and de-noised by utilization of Daubechies Wavelet of order 3, as seen in Figure 6. The total local maxima of the curve is recorded as the total cell count for that layer of the sample. The original Optical Quadrature image is then analyzed to determine whether a second layer of cells exists and requires counting. The minimum cell size and single cell threshold are calculated based on the total area of the sample in the first count. If the area of points above the second layer threshold is above the minimum cell size and below the single cell threshold one cell is added to the total cell count. However, if the area of points above the second layer threshold is above the single cell threshold, the contour signature method is repeated.

A program utilizing this method has been produced with two sets of experimentally chosen threshold values for a low number of cells, less than 10, and a high number of cells, more than 10. Both sets of threshold values were applied to a group of 67 embryos ranging in cell number from eight to thirty one. For samples with less than 10 cells the failure rate, depicting an inability to produce a count, was 9.09% with a rms error of 1.64 cells. For samples with more than 10 cells, the failure rate for the best set of threshold values was 19.64% with a rms error of 6.89 cells. The full results can be seen in Table 1.

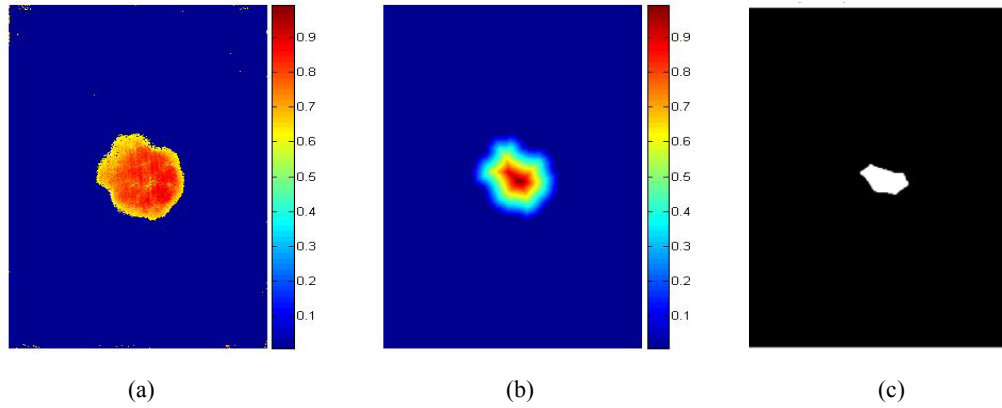


Figure 5: (a) Normalized, edge-enhanced Optical Quadrature image, (b) Distance transform, (c) Binary image of distance transform from experimentally chosen threshold.

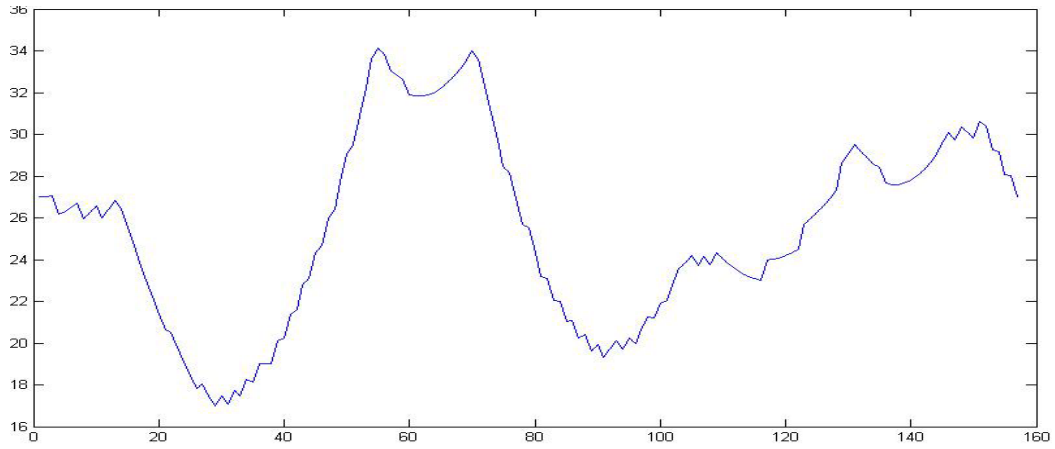


Figure 6: Contour signature curve after smoothing with six local maxima

	<b>Sample Size</b>		<b>Failure Rate</b>	<b>Error</b>
<b>6 – 10 cells</b>	11 Samples	Threshold 1	18.18%	$\pm 9.2$ cells
		Threshold 2	9.09%	$\pm 1.64$ cells
<b>10 – 16 cells</b>	29 Samples	Threshold 1	27.59%	$\pm 5.54$ cells
		Threshold 2	3.45%	$\pm 7.24$ cells
<b>16 – 31 cells</b>	27 Samples	Threshold 1	11.11%	$\pm 7.88$ cells
		Threshold 2	14.81%	$\pm 16.61$ cells
<b>10 – 31 cells</b>	56 Samples	Threshold 1	19.64%	$\pm 6.89$ cells
		Threshold 2	8.93%	$\pm 12.38$ cells

Table 1: Results of Contour Signature Method on 67 embryos.

## 8. FUTURE WORK

To improve accuracy using a model based algorithm, we have developed an elliptical fit cell counting algorithm that uses the combination of registered DIC and Optical Quadrature images. This algorithm takes advantage of the distinct cell boundaries within the DIC image for cells within the focal plane. A parabolic fit is created for a non-overlapped region of one cell and the maximum is set as the maximum optical path difference. The user then fits an elliptical boundary around a cell on the DIC image and it is used with the maximum optical path difference to create an ellipsoid representing that particular cell. The ellipsoid is subtracted from the Optical Quadrature image leaving either the background or the cells outside of the focal plane not seen in the DIC image. The elliptical boundary procedure is repeated until only the background remains in the subtracted Optical Quadrature image. This algorithm has only been used on a few samples but has shown great potential.

## ACKNOWLEDGEMENTS

This work was supported in part by CenSSIS, the Center for Subsurface Sensing and Imaging Systems, under the Engineering Research Centers Program of the National Science Foundation (Award Number EEC-9986821).

## REFERENCES

1. Steptoe, P., Edwards, R., Purdy, J., "Clinical aspects of pregnancies established with cleaving embryos grown in vitro," *Br. J. Obstet. Gynaecol.*, **87**, 757-768, 1980.
2. Dean, N., Philips, S., Buckett, W., Biljan, M., Lin Tan, S., "Impact of reducing the number of embryos transferred from three to two in women under the age of 35 who produced three or more high-quality embryos," *Fertil. Steril.*, **74**, 820-823, 2000.
3. Fujii, S., Fukui, A., Yamaguchi, E., Sakamoto, T., Sato, S., Saito, I., "Reducing multiple pregnancies by restricting the number of embryos transferred to two at the first embryo transfer attempt," *Hum. Reprod.*, **13**, 3550-3554, 1998.
4. Hu, Y., Maxson, W., Hoffman, D., Ory, S., Eager, S., Dupré, J., Lu, C., "Maximizing pregnancy rates and limiting higher-order multiple conceptions by determining the optimal number of embryos to transfer based on quality," *Fertil. Steril.*, **69**, 650-657, 1998.
5. Milki, A., Fisch, J., Behr, B., "Two-blastocyst transfer has similar pregnancy rates and a decreased multiple gestation rate compared with three-blastocyst transfer," *Fertil. Steril.*, **72**, 225-228, 1999.
6. Nijs, M., Geerts, L., van Roosendaal, E., Segal-Bertin, G., Vanderzwalmen, P., Schoysman, R., "Prevention of multiple pregnancies in an in vitro fertilization program," *Fertil. Steril.*, **59**, 245-250, 1993.
7. Staessen, C., Janssenswillen, C., Van den Abeel, E., Devroey, P., Van Steirteghem, A., "Avoidance of triplet pregnancies by elective transfer of two good quality embryos," *Hum. Reprod.*, **8**, 1650-1653, 1993.
8. Templeton, A., Morris, J., "Reducing the risk of multiple births by transfer of two embryos after in vitro fertilization," *N. Engl. J. Med.*, **339**, 573-577, 1998.
9. Gerris, J., De Neubourg, D., Mangelschots, K., Van Royen, E., Vercruyssen, M., Barudy-Vasquez, J., Valkenburg, M., Ryckaert, G., "Elective single day 3 embryo transfer halves the twinning rate without decrease in the ongoing pregnancy rate of an IVF/ICSI programme," *Hum. Reprod.*, **17**, 2626-2631, 2002.
10. De Neubourg, D., Mangelschots, K., Van Royen, E., Vercruyssen, M., Ryckaert G., Valkenburg, M., Barudy-Vasquez, J., Gerris, J., "Impact of patients' choice for single embryo transfer of a top quality embryo versus double embryo transfer in the first IVF/ICSI cycle," *Hum. Reprod.*, **17**, 2621-2625, 2002.
11. ESHRE Campus Course Report, "Prevention of twin pregnancies after IVF/ICSI by single embryo transfer," *Hum. Reprod.*, **16**, 790-800, 2001.
12. Martin, J., Park, M., "Trends in twin and triplet births: 1980-1997," *Natl. Vital Stat. Rep.*, **24**, 1-16, 1999.
13. Reynolds, M., Schieve, L., Martin, J., Jeng, G., Macaluso, M., "Trends in Multiple Births Conceived Using Assisted Reproductive Technology, United States, 1997-2000," *Pediatrics*, **111**, 1159-1162, 2003.
14. Gardner, M., Goldenberg, R., Cliver, S., Tucker, J., Nelson, K., Copper, R., "The origin and outcome of preterm twin pregnancies," *Obstet. Gynecol.*, **85**, 553-557, 1995.

15. Guyer, B., Martin, J., MacDorman, M., Martin, J., Peters, K., Strobino, D., "Annual summary of vital statistics – 1996," *Pediatrics*, **100**, 905-918, 1997.
16. Kiely, J., "What is the population-based risk of preterm birth among twins and other multiples?" *Clin. Obstet. Gynecol.*, **41**, 3-11, 1998.
17. Kinzler, W., Ananth, C., Vintzileos, A., "Medical and economic effects of twin gestations," *J. Soc. Gynecol. Investig.*, **7**, 321-327, 2000.
18. Pharoah, P Cooke, T., "Cerebral palsy and multiple births," *Arch. Dis. Chil. Fetal. Neonatal. Ed.*, **75**, F174-F177, 1996.
19. Senat, M., Ancel, P., Bouvier-Colle, M., Breart, G., "How does multiple pregnancy affect maternal mortality and morbidity?" *Clin. Obstet. Gynecol.*, **41**, 78-83, 1998.
20. Spellacy, W., Handler, A., Ferre, C., "A case-control study of 1,253 twin pregnancies from a 1982-1987 perinatal data base," *Obstet. Gynecol.*, **75**, 168-171, 1990.
21. Van Royen, E., Mangelschots, K., De Neubourg, D., Valkenburg, M., Van de Meerssche, M., Ryckaert, G., Eestermans, W., Gerris, J., "Characterization of a top quality embryo, a step towards single embryo transfer," *Hum. Reprod.*, **14**, 2345-2349, 1999.
22. Gurgan, T., Demirel, A., "Why and how should multiple pregnancies be prevented in assisted reproduction treatment programmes?" *Reprod. Biomed. Online*, **9**, 237-244, 2004.
23. Gardner, D., Lane, M., "Culture and selection of viable blastocysts: a feasible proposition for human IVF?" *Hum. Reprod.*, **3**, 367-382, 1997.
24. Glina, Y., Tsihrintzis, G., Warner, C., Hogenboom, D., DiMarzio, C., "On the use of the optical quadrature method in tomographic microscopy," *Bios*, **3605**, 1999.
25. Ghiglia, D., Pritt, M., *Two-Dimensional Phase Unwrapping: Theory, Algorithms, and Software*, 248-273, John Wiley and Sons, New York, 1998.

\*wwarger@ece.neu.edu, Ph: 617 373 7756, Fax: 617 373 7783