



Recommended Contrasting Techniques in Assisted Reproductive Technologies

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Author: Carl Zeiss Microscopy GmbH, Germany

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Introduction

Historically, microscopy has played an important role in our understanding of the morphology and role of sperm and ova in reproduction, elucidating their behavior before and during their fusion, and helping us understand the early stages of zygote development. Assisted reproductive technology (ART) has undergone rapid development (figure 1) since the introduction of *in vitro* fertilization (IVF) in the late 1970s.

IVF, intra-cytoplasmic sperm injection (ICSI), and intra-cytoplasmic morphologically selected sperm injection (IMSI) are reproductive techniques where ova are fertilized *in vitro* with sperm cells. The resulting fertilized eggs (zygotes) are then implanted into the uterus to establish pregnancy. Microscopy is an essential component of these ART procedures, providing detailed visualization of gametes and zygotes, permitting

the assessment of key clinical parameters, and allowing embryologists to perform micromanipulation techniques without adversely affecting the viability of cells or embryos. Appropriate transmitted light microscopy contrast techniques that are specialized for live and unstained samples are essential in the field of ART.

In microscopy, differences in intensity and/or color create contrasts that allow individual features and details of the specimen to become visible. As a general rule, a contrast value of no less than 2 % is required for the human eye to distinguish an object from background, or a detail from the rest of the structure. This value may differ for other detectors such as video cameras, or photodetectors. Single live cells are essentially colorless and transparent making them hard

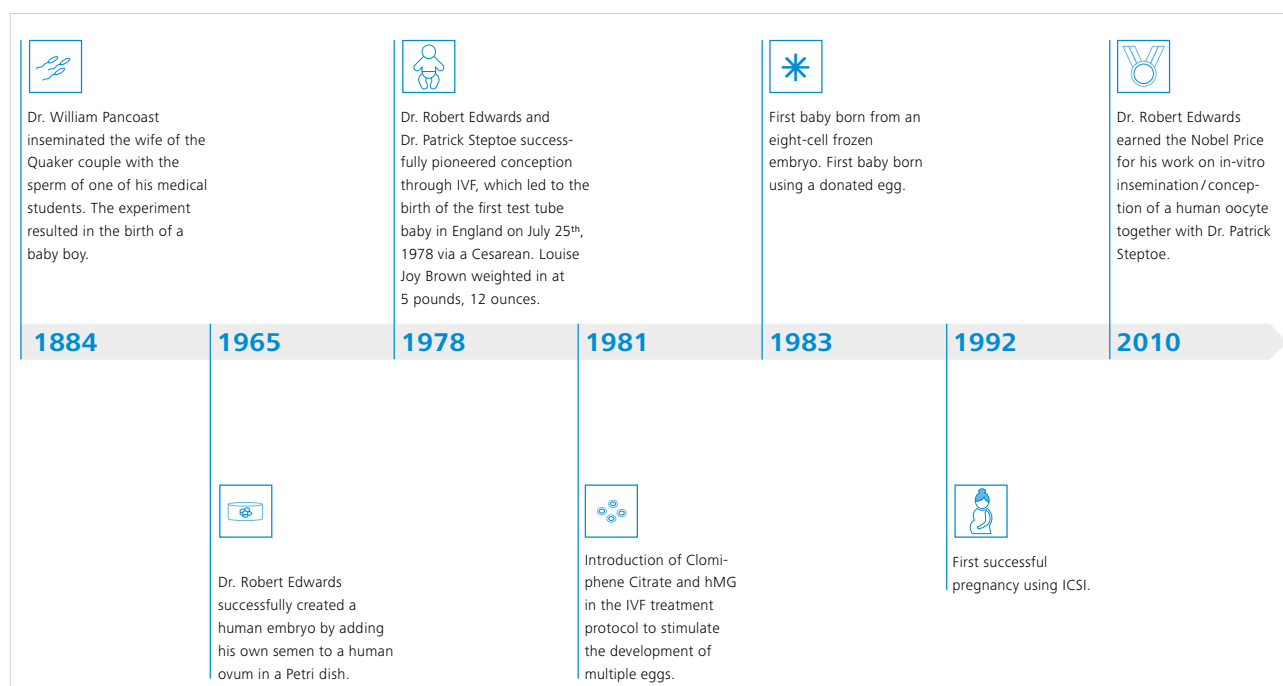


Figure 1 Main steps in development of ART

to visualize. Contrast enhancing dyes cannot be employed in ART as these would compromise the health and viability of the cells. However, absorbance is not the only way light can interact with the cell. Other phenomena such as light scattering, and diffraction are also used in microscopy to realize optimum contrast without affecting cellular health. ART laboratories are therefore equipped with a wide range of compound and stereo microscope systems with various contrast-creating techniques.

Phase Contrast Microscopy

Phase contrast microscopy effectively translates small phase variations into corresponding amplitude differences, creating visible image contrast. This technique is ideal for thin, unstained specimens, such as cultured cells in a Petri dish that are approximately 5 to 10 micrometers thick.

In a phase contrast microscope, the condenser contains an annulus called the 'phase stop' that produces a hollow cone of light that illuminates the specimen at the focal point. As light passes through the object, the rays are deflected from their original direction. The size of the phase stop depends upon the objective and condenser numerical aperture (NA).

Light enters the objective and an image of the phase stop is created in the rear focal plane, known as the objective pupil. Positioned within the rear focal plane of the objective is a phase ring that is aligned with the condenser phase stop.

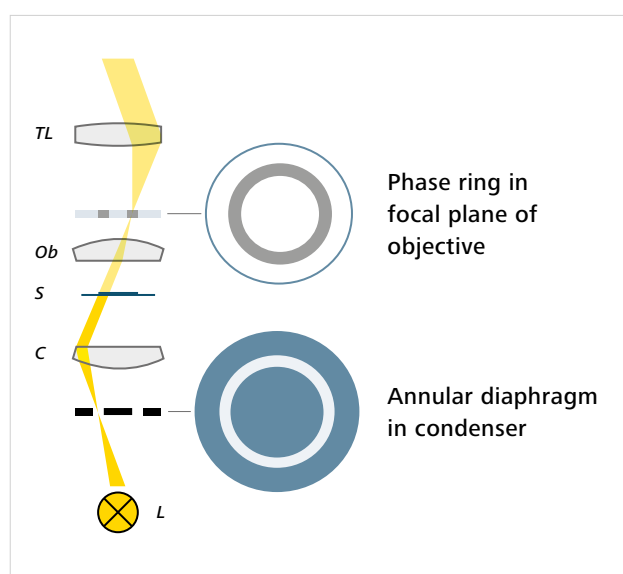


Figure 2 Principle of phase contrast
Beam path: (L – Light source, C – Condenser, S – Specimen, Ob – Objective, TL – Tube lens)

The light that passes through the specimen is usually retarded by a quarter of a wavelength, whereas background light is usually advanced by a quarter of a wavelength. This results in a difference between deviated (passing through the specimen) and undeviated (background) light of up to half a wavelength (figure 2). Although the human eye is unable to distinguish phase shifts, the phase contrast microscope translates these differences into visible changes in amplitude/intensities, resulting in contrast details that appear darker against a light background.

Phase contrast is an excellent method to increase contrast when imaging live, unstained cells. However, phase images are often surrounded by bright halos that appear on the borders surrounding the specimen and contrasting details. These occur because the circular phase-retarding neutral density ring in the objective phase plate also transmits some diffracted light from the specimen. The technique is not useful for the visualization of thick specimens, as they often have highly overlapping structures, producing more severe halo artefacts. In ART, phase contrast is often used for sperm visualization and the evaluation of sperm motility.

The table below highlights some of the advantages and disadvantages of phase-contrast microscopy:

Advantages	Disadvantages
■ High contrast	■ Halo artefacts
■ Good resolution	■ Annuli can limit the aperture and decrease resolution
■ Works well for thin samples	■ Not suitable for thick specimens
■ Inexpensive	
Recommended for:	
■ Sperm visualization	
■ Evaluation of sperm motility	

Table 1 Advantages and disadvantages of phase contrast microscopy

Differential Interference Contrast (DIC) Microscopy

DIC microscopy is capable of delivering sharp, detailed images to visualize fine structures in cells and tissues by using polarized light to detect refractive index and thickness differences in specimens. Most brightfield microscopes can be retrofitted with the components necessary to employ this imaging technique. The necessary elements of a DIC system (figure 3) are:

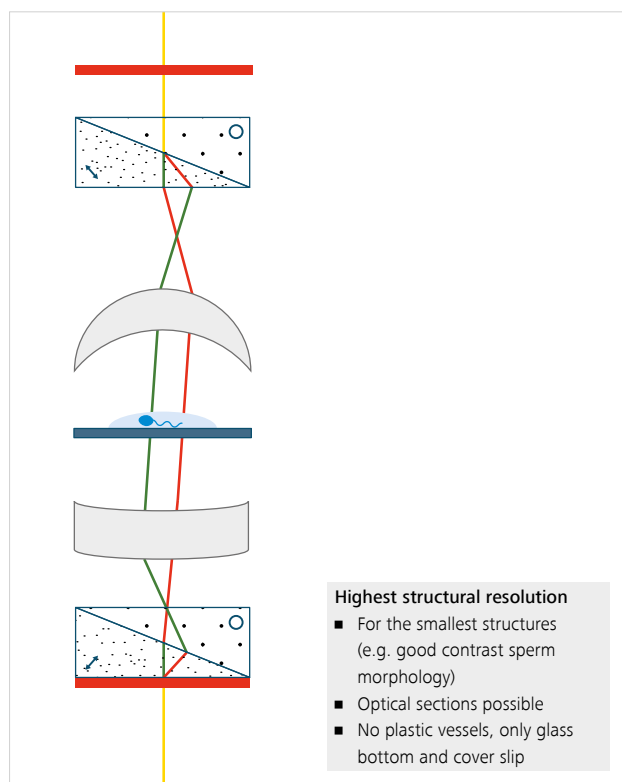


Figure 3 Principle of Differential Interference Contrast (DIC) Microscopy

- A polarizer inserted between the illumination source and the condenser that produces linearly polarized light.
- A Nomarski prism used to split the linearly polarized light into two beams.
- An objective prism (a second Nomarski prism) for recombining the two separated beams.
- An analyzer, which is a second polarizing filter usually located behind the objective prism and oriented perpendicular to the transmission path of the first polarizer. This is where the interference occurs that generates the DIC image.

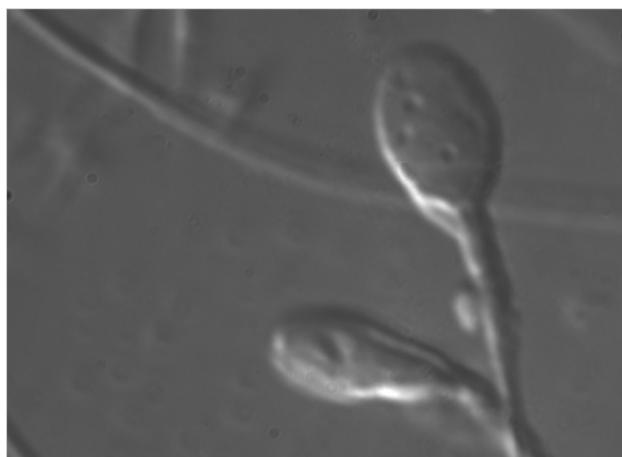


Figure 4 Sperm cells acquired with DIC

The sample is effectively illuminated by two split beams with orthogonal polarization that are spatially slightly displaced (sheared). The two wavefronts travel through adjacent areas of the sample. If the two beams see a difference in refractive index or specimen thickness they experience different optical path lengths which results in changes of the phase difference of the two wavefronts. At the objective prism the two beams are recombined before they travel through an analyzer where interference due to the phase difference occurs. The resulting DIC image is formed by converting phase differences in visible changes of brightness. The sample appears as pseudo-3D relief which should not be taken as a representation of the actual topography.

DIC microscopy provides high resolution images of unstained biological samples (figure 4), and its primary advantage over phase contrast is the ability to utilize the instrument at full NA without the light-reducing effects of phase plates or condenser annuli. Although DIC microscopy is the main technique used for the morphological grading of sperm cells in IMSI, it requires glass-bottom culture plates (to preserve the polarization of light) along with high magnification and high NA oil objectives.

The table below highlights some of the advantages and disadvantages of DIC microscopy:

Advantages	Disadvantages
<ul style="list-style-type: none"> ■ Provides high resolution and contrast, even with thick specimens ■ No halo artefacts ■ Can be combined with fluorescence microscopy without a loss of signal 	<ul style="list-style-type: none"> ■ Requires glass bottom culture dishes (therefore incompatible with routine culture plastics) ■ High setup and running cost
Recommended for:	
<ul style="list-style-type: none"> ■ Morphological grading of sperm cells in IMSI 	

Table 2 Advantages and disadvantages of DIC microscopy

PlasDIC Microscopy

PlasDIC microscopy was introduced by ZEISS as an innovative contrast technique for routine observation of live, unstained specimens, including gametes and embryos. As the name implies, it is a variation of DIC microscopy that is appropriate for use with plastic-bottom culture containers that are not only cheaper, but offer a less hostile growing environment than glass. While PlasDIC uses the same principles as DIC

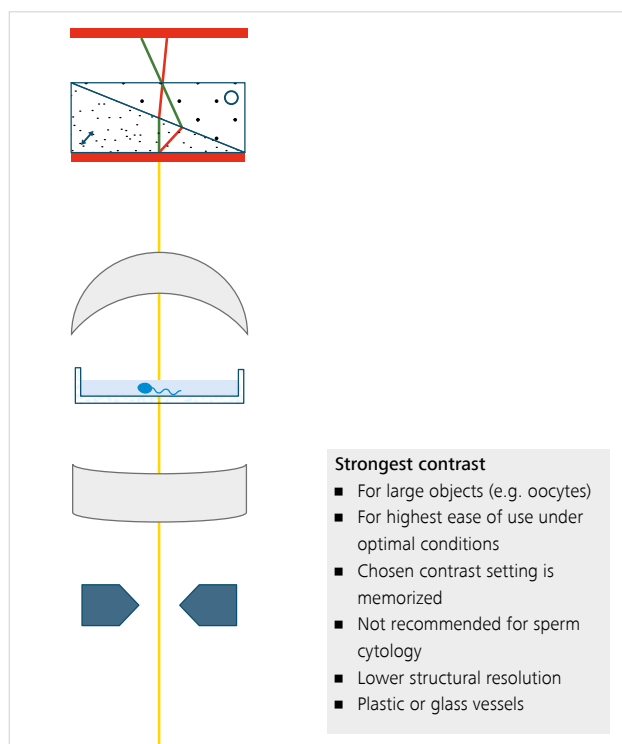


Figure 5 Principle of PlasDIC Microscopy

microscopy, the sample is instead illuminated with non-polarized light, and both the polarizer and Nomarski prism are placed after the birefringent specimen has induced a phase distortion to the wavefront. The Nomarski prism splits the wavefront in two and a downstream analyzer oriented at 45° allows only the transmission of light that oscillates in the same plane and can therefore interfere. Interference then occurs when the two wavefronts become coherent at the intermediate image plane. As the specimen and culture container lie outside the area sensitive to polarization, the presence of plastic has no adverse effect on the image.



Figure 6 Oocyte acquired with PlasDIC

PlasDIC microscopy delivers crisp relief contrast and an impressive pseudo-3D image (figure 6). Details of the zona pellucida, oolemma and also the pipettes can be visualized nicely. Therefore, PlasDIC is especially suited for ICSI as standard plastic labware can be used.

The table below highlights some of the advantages and disadvantages of PlasDIC microscopy:

Advantages	Disadvantages
<ul style="list-style-type: none"> ■ Relatively inexpensive ■ Simple setup ■ Highest ease of use – when switching magnifications, condensor position stays the same; no switch necessary ■ No halo artefacts ■ High contrast DIC-like image, even with standard plastic dishes 	<ul style="list-style-type: none"> ■ Slit diaphragm reduces available illumination ■ Lower structural resolution compared to DIC
Recommended for:	
<ul style="list-style-type: none"> ■ ICSI as standard plastic labware can be used ■ Good visualization of the zona pellucida, oolemma and pipettes 	

Table 3 Advantages and disadvantages of PlasDIC microscopy

Hoffman Modulation Contrast Microscopy

Hoffman modulation contrast (HMC) microscopy is an oblique illumination technique that achieves enhanced contrast in live specimens by converting phase gradients in the sample structure into amplitude (or brightness) changes and creating pseudo-3D images of unstained samples. The Hoffman modulator, which is an optical amplitude spatial filter, is inserted in the back focal plane of the objective. The modulator has three zones: (1) a small, dark zone transmitting a fixed low percentage of light; (2) a narrow gray zone with 15 % light transmission; and (3) the remaining clear or transparent zone, transmitting 100 % of the light. Unlike the phase plate used in phase contrast microscopy, the modulator does not introduce any change to the phase of the light passing through these zones. Coupled to the objective modulator is an off-axis slit aperture placed in the condenser front focal plane, directing oblique illumination towards the specimen. The effective slit width can be modulated by a polarizer. The slit is adjusted so that light transmission through the slit falls on the gray region (15 %) of the modulator. Light that passes through homogeneous areas within the sample will not be

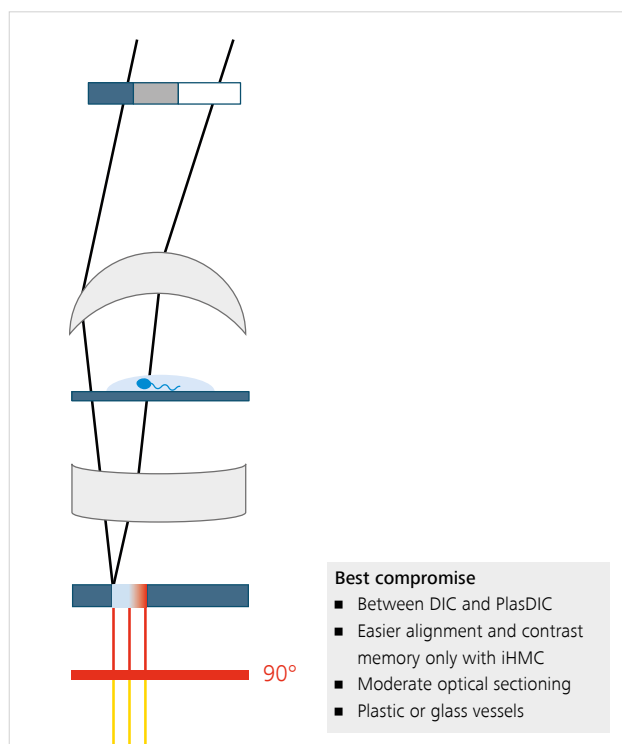


Figure 7 Principle of Hoffman Modulation Contrast Microscopy

refracted, but will pass through the central gray region of the modulator and be rendered gray. In regions of the sample where a structure causes an optical phase gradient, refraction will occur; the path of the refracted light will be shifted either towards the transparent or the dark zone of the modulator. This results in a pseudo-3D image appearing. Unlike DIC microscopy, HMC uses no beam-splitting prisms and the two polarizers are mounted optically before the sample; thus, HMC can be used in combination with birefringent specimens and materials such as plastic Petri dishes. The improved HMC (iHMC) from ZEISS delivers a crisp relief contrast and reveals even finest structures in the cell nucleus, the nucleus shape, and nucleoli (figure 8).

HMC is the most widely used contrast technique in ART using inverted microscopes as it offers a good compromise between resolution and contrast. This technique is routinely used for ICSI and embryo quality assessment, and is also suitable for quick sperm morphology examinations.

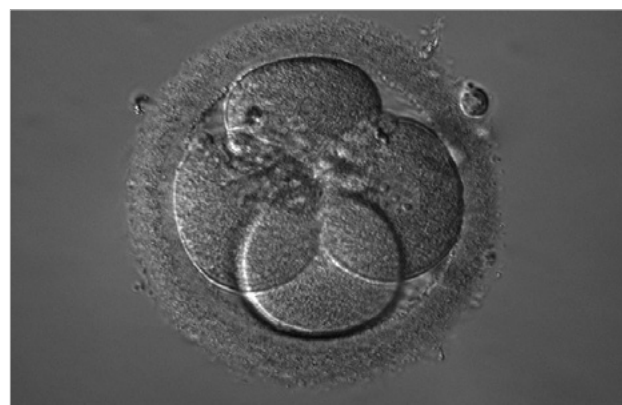


Figure 8 Embryo image acquired with iHMC

Table 4 highlights some of the advantages and disadvantages of HMC microscopy.

Advantages	Disadvantages
<ul style="list-style-type: none"> ■ Suitable for thin or thick specimens ■ 'Contrast memory' from ZEISS makes alignment easier ■ Compatible with standard plastic dishes ■ No halo artefacts ■ Offers good compromise of resolution and contrast 	<ul style="list-style-type: none"> ■ Higher setup costs than PlasDIC ■ Magnification changes also require a diaphragm change on the condenser
Recommended for:	
<ul style="list-style-type: none"> ■ ICSI ■ Embryo quality assessment ■ Fast sperm morphology examination 	

Table 4 Advantages and disadvantages of HMC microscopy

Summary of ART Contrast Techniques

In conclusion, there are a variety of contrast techniques available for use in reproductive medicine today, each suited for certain applications, addressing the particular requirements of both the sample and the job to be done.

Microscopy Technique	Main Applications	Dish material
Phase Contrast	Sperm counting, evaluation of sperm motility	Glass/plastic
DIC	IMSI	Glass
PlasDIC	ICSI	Plastic/glass
iHMC	ICSI	Plastic/glass

References:

- [1] Kashir J, Jones C, Ramadan W, Kang YJ, Carver J, Griffiths T, Turner K, Coward K (2012) Magnifying human fertility: microscopy and assisted reproductive technology. *InFocus*, 25, 23–41
- [2] [In Vitro Fertilization and Reproductive Medicine](#) A summary of IVF, ICSI and IMSI with recommended microscope systems for each ART technique.



Carl Zeiss Microscopy GmbH
07745 Jena, Germany
microscopy@zeiss.com
www.zeiss.com/ivf



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