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# Confocal Scanning Fluorescence Microscopy: A New Method for Phagocytosis Research

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**An important new method for phagocytosis research, confocal scanning fluorescence light microscopy (CSFM), is demonstrated using fluorescent microspheres ingested by murine macrophages. CSFM, in combination with Nomarski differential interference contrast microscopy (DIC), can resolve microspheres inside cells from microspheres attached to the surface of cells. Further, combined CSFM and DIC images can quantitate phagocytosis by individual cells aggregated together. No other method offers these capabilities. A comparison of CSFM and conventional epifluorescence light microscopy (EFM) images shows that CSFM produces significantly higher-resolution images of microspheres than EFM, primarily because CSFM excludes the out-of-focus light artifacts of EFM.**

**Key words:** imaging, fluorescent microspheres, macrophages

## INTRODUCTION

Since the pioneering work of Metchnikoff, the light microscope has been an essential tool for phagocytosis research [7]. More recently, epifluorescence light microscopy (EFM) has become a valuable method for quantitating the number of fluorescent microspheres ingested by individual macrophages [2,4]. However, the resolution of EFM is limited by blurs and halos caused by light from fluorescent structures located below or above the plane of focus which causes several severe limitations in phagocytosis research. Blurs and halos make it impossible to determine if fluorescent microspheres are in or on individual macrophages and EFM cannot quantitate directly phagocytosis [1,10]. Further, the out-of-focus artifact is compounded when cells are aggregated together and EFM cannot determine phagocytosis of individual cells within cell clusters. Various sample preparation methods—such as cytocentrifugation [4], washing [9], and extracellular fluorescence quenching [3]—effectively eliminate extracellular microsphere fluorescence, but these methods do not alter the fundamental limitations caused by out-of-focus light artifacts.

Now, a new fluorescence microscopy method, confocal scanning fluorescence microscopy (CSFM), has been developed that excludes all out-of-focus light and eliminates the halos and blurs of conventional fluorescent images [11]. Even though CSFM has several important optical advantages over EFM, CSFM has not been applied to phagocytosis research. This paper demonstrates the utility of CSFM for phagocytosis research

by using it to study fluorescent microspheres ingested by murine macrophages.

## MATERIALS AND METHODS

### Confocal Scanning Fluorescent Microscopy

Figure 1 is a diagrammatic representation of a CSFM system, which consists of a laser, mirrors, light microscope, filter cube, pinhole aperture, detector, computer, and cathode-ray tube. A laser (argon ion) beam forms a small spot (0.2  $\mu\text{m}$  diameter) that is scanned in a raster pattern by synchronized rocking mirrors. A filter cube selects the excitation frequencies and reflects the scanning beam into the objective lens of a light microscope, which focuses it onto the sample. The scanning beam causes fluorescent light to be emitted from the fluorochrome in the sample. The fluorescent light travels back through the objective lens to the filter cube where a barrier filter passes it through to a pinhole aperture. The pinhole aperture (40–50  $\mu\text{m}$  diameter) is the key component to the confocal system because it blocks all out-of-focus light (Fig. 2) and acts as a spatial filter for the third dimension of the sample (the third dimension is that which is orthogonal to the plane of focus). The fluorescent light from the plane of focus passes through the pinhole aperture and is detected by a photomultiplier

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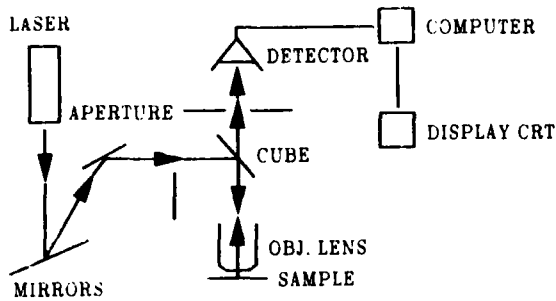


Fig. 1. A schematic diagram of a CSFM system. The laser forms a small beam spot and the rocking mirrors cause the beam to scan a raster pattern. The epi-illumination filter cube selects the excitation frequency and the objective lens focuses the excitation beam on the sample. The sample emits fluorescent light, which travels back through the objective lens and filter cube, which discriminates the emitted fluorescent light. The pinhole aperture excludes all out-of-focus light and the detector converts the light to a video signal. A computer digitizes the video signal, which is displayed on the cathode-ray tube.

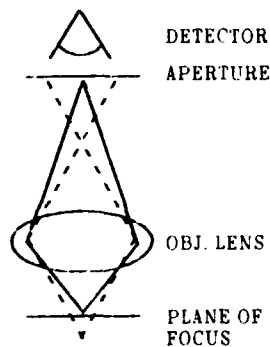


Fig. 2. A schematic diagram showing how the pinhole aperture spatially filters the image in the third dimension. Light from the plane of focus (solid ray lines) is focused on the pinhole aperture and passes through, whereas light from the out-of-focus plane (dashed ray lines) is not focused on the pinhole aperture and is blocked. Thus, only light from the plane of focus is detected using CSFM.

tube, which converts the light into a video signal. The video signal is digitized in a computer and displayed on a cathode-ray tube.

The CSFM images are created in time by the scanning beam. Approximately 1 or 2 sec per scan are required with several scans usually needed to achieve an image with a good signal-to-noise ratio. Since the microspheres are extremely bright and do not bleach, a 2-sec total acquisition time was sufficient to obtain CSFM images of microspheres.

We used the BioRad-Laserssharp MRC500 (Cambridge, MA) and the Zeiss LSM (Thornwood, NY). The BioRad-Laserssharp system can be attached to any upright or inverted light microscope, whereas the Zeiss

LSM system uses a built-in, dedicated upright microscope. We used Zeiss planapochromat objective lenses ( $63\times$ , n.a. 1.4 or  $40\times$ , n.a. 0.9) and DIC optics for both systems. Both CSFM systems used an IBM compatible computer, and digital images were displayed in 256 gray levels or pseudocolor.

Nonconfocal EFM images were obtained by removing the pinhole aperture. The same scanning laser, filter cube, detector, display, image averaging, and gray levels were used for both the laser EFM and CSFM images.

### Nomarski Differential Interference Contrast Microscopy

Although not confocal, DIC also only images the plane of optical focus and as a result is useful for high-resolution imaging of cell structure in cell aggregates [5]. DIC images were made simultaneous with CSFM images. The transmitted laser light was routed via a light pipe to a second photomultiplier tube. The light signal was converted to video, digitized, and displayed on a cathode-ray tube in the same way as the CSFM images. Combined digital CSFM and DIC images were 8 bits total with each component image being 4 bits. Conventional DIC optics, Wollaston calcium calcite beam-splitting prisms, and a condenser polarizer were required. The cells must be mounted on glass and not plastic because plastic depolarizes light but glass does not.

### Photography

CSFM, EFM, and DIC images were photographed from either a high-resolution black and white or color cathode-ray tube with Kodak Panatomic-X or Kodak Gold 100 35-mm film, respectively. The CSFM and EFM images were processed identically and the black and white film was developed using Kodak Microdol-X and printed on Agfa 1-1 paper. The color film was commercially developed and printed.

### Macrophage Culture

We euthanized four BALB/c female mice by cervical dislocation, lavaged their peritoneal cavities with Hanks' balanced salt solution (Gibco, Grand Island, NY), and pooled the peritoneal exudate cells in RPMI 1640 medium (GIBCO, Grand Island, NY) with 10% fetal calf serum, 2 mM glutamine and gentamicin sulfate. We equally distributed the cells among four plastic petri dishes (60-mm diameter, Costar, Cambridge, MA), each containing a sterile cover glass (24-mm diameter, No. 1.5). We incubated the cells at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  overnight and washed away nonadherent cells. To the cultures we added fluorescein conjugated, 1.16- $\mu\text{m}$  diameter, carboxylate microspheres (Polysciences, War-

rington, PA) at a cell-to-bead ratio of 1:100. We incubated the cells and beads together for 1 hr, decanted the medium, and fixed the cells with 2% paraformaldehyde in isosmotic phosphate-buffered saline. We removed the cover glasses from the dishes, wiped dry the backside of the cover glasses, placed the cover glasses cell side down in glycerol on glass slides, and glued the edges of the cover glasses to the slides with clear nail polish.

## RESULTS

Figure 3 is a direct comparison of CSFM and EFM images and shows that CSFM produces significantly higher-resolution images of the microspheres than EFM. CSFM images show the fluorescent microspheres only in the optical planes that contain the microspheres, whereas EFM images show fluorescent halos and blurs in optical planes that do and do not contain the microspheres. Thus, CSFM provides the precise three-dimensional location of microspheres but EFM does not.

Digitally combined CSFM and DIC images can accurately determine the subcellular position of the microspheres. CSFM and DIC images can be accurately combined because the two images are obtained in parallel and have exactly the same registration. Digital CSFM and DIC images are assigned different pseudocolors so that the contribution of each image can be determined when the two images are superimposed. Figure 4 shows digitally combined CSFM and DIC images of macrophages that have ingested microspheres. The CSFM portion of each image is displayed in yellow, and the DIC portion of the image is in green. The four images are of four different optical sections with each image being the result of combining a different pair of CSFM and DIC images. Microspheres inside and attached to the outside of the cells can be seen in these combined CSFM and DIC images.

The four images in Figure 4 are part of a complete set of 12 optical sections through the entire cell aggregate. From a complete set of combined CSFM and DIC serial sections, the number of microspheres inside each macrophage was counted. Figure 5 shows the number of microspheres in each cell and the position of each cell relative to Figure 4. This result demonstrates that phagocytosis by individual cells clumped together can be quantitated using a through-focus series of digitally combined CSFM and DIC images.

## DISCUSSION

Combined CSFM and DIC images can resolve a fundamental problem in phagocytosis research, namely, whether microspheres are inside or attached to macro-

phages. Fluorescent-activated cell-sorter analysis (FACS) is an alternative method for quantitating phagocytosis by individual cells [6] and can rapidly analyze many cells but FACS, like EFM, cannot determine if microspheres are inside or attached to individual macrophages. Thus, CSFM and FACS provide complementary information on individual cells and cell populations, respectively.

Combined CSFM and DIC imaging provides exciting opportunities for studying phagocytosis by cells in aggregates. Previously, phagocytosis within aggregated cells could not be studied and, now, questions concerning the effects of cell-cell interactions on phagocytosis can be addressed using combined CSFM and DIC imaging. Phagocytosis by cells in aggregates may be different from phagocytosis by isolated cells and may be a better model of *in vivo* phagocytosis.

Combined CSFM and DIC imaging also is useful for studying the fate and traffic of ingested particles because the subcellular location of fluorescent particles can be rapidly determined. The alternative method, transmission electron microscopy, is significantly slower than CSFM and cannot routinely reconstruct the entire cell.

CSFM provides superior three-dimensional resolution and light sensitivity over EFM for several reasons. Because resolution is not limited by out-of-focus light, CSFM resolution in the third dimension is only limited by the depth ( $D$ ) of the in-focus optical plane, which is given by

$$D = W/(N.A.)^2,$$

where  $W$  is the wavelength of light, and N.A. is the numerical aperture of the objective lens. Using a 488-nm light source and a 1.4 N.A. objective lens, the resolution of CSFM in the third dimension is theoretically 0.3  $\mu\text{m}$ . CSFM also has a theoretical 1.4-fold better lateral resolution than EFM [8]. CSFM has a higher sensitivity for low light fluorescence than EFM because the laser light source has higher intensity than conventional light sources, the CSFM photomultiplier detector has greater sensitivity than film, and, most important, computer image enhancement, especially image averaging, increases the signal-to-noise ratio of CSFM images.

There are several problems inherent to CSFM. CSFM does not allow real-time recording because CSFM images take several seconds to acquire and rapidly occurring events cannot be studied in live cells. Further, the high intensity of the laser may have deleterious effects on live cells. Finally, CSFM is technically and operationally more complicated as well as significantly more expensive than conventional EFM.

In conclusion, a new method—CSFM—offers many exciting opportunities for phagocytosis research.

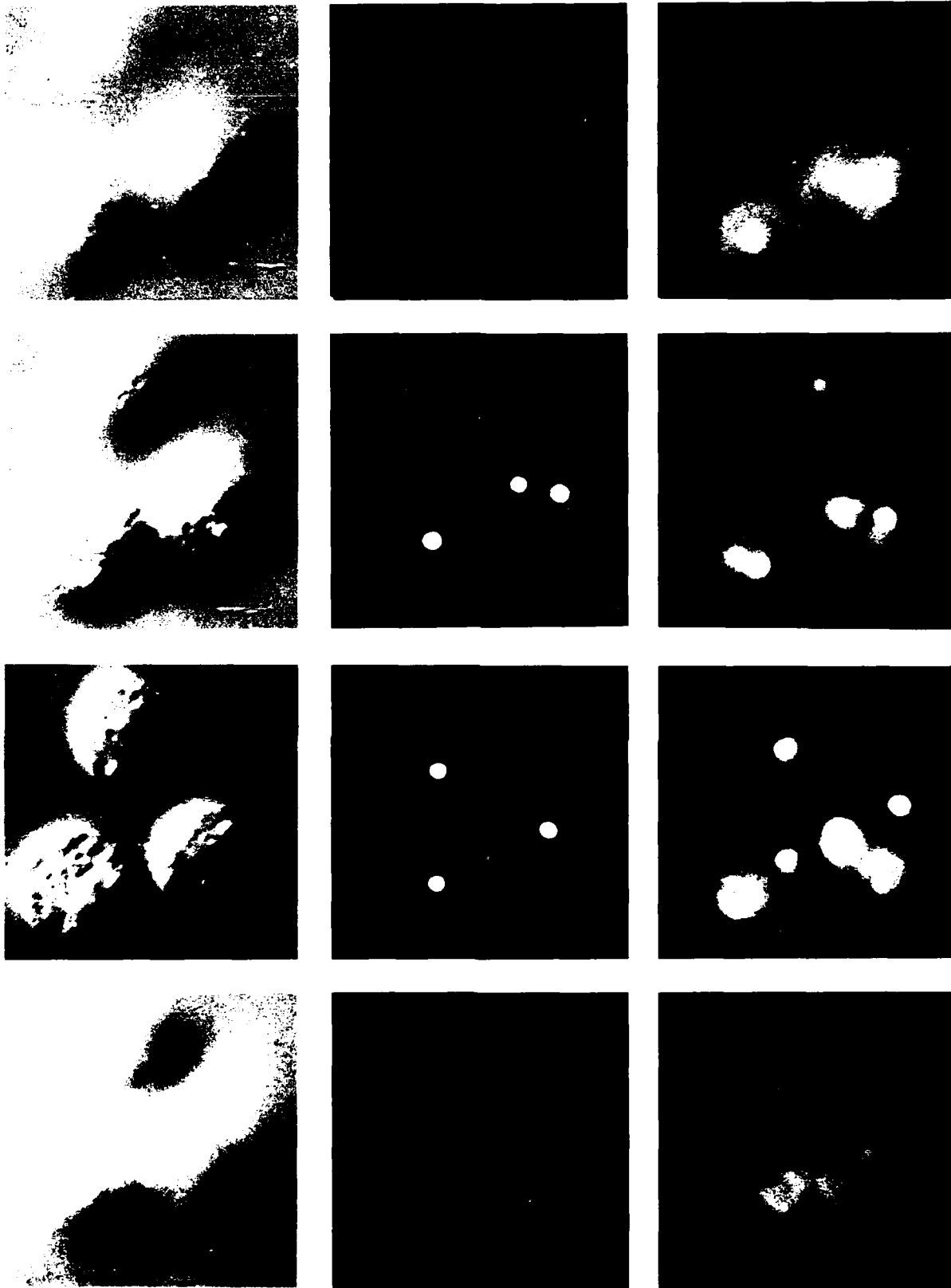
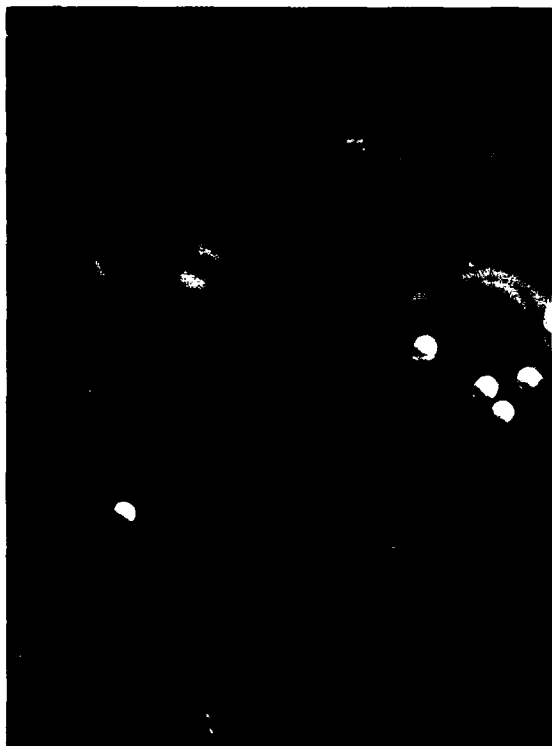


Fig. 3. DIC (left column), CSFM (middle column), and EFM (right column) images are shown of optical sections (rows) through three macrophages that have ingested fluorescent microspheres. From the top to bottom of the figure the optical sections are: row 1, through the top surface of two cells; row 2, through the middle of two cells and the top surface of a third cell; row 3, through the middle of all three cells; row 4, through the attachment of the cells to the glass. It is clear from

a comparison of the CSFM and EFM images from the same optical plane that the CSFM images do not have halos and blurs whereas the EFM images do. Only microspheres in the plane of focus are imaged by CSFM, whereas microspheres in, below, and/or above the plane of focus are imaged by EFM. It is possible to accurately locate the microspheres in three dimensions by CSFM but not by EFM. (Full picture width = 16  $\mu\text{m}$ ).



**Fig. 4.** Four digitally combined DIC (green) and CSFM (yellow) optical sections through six macrophages are shown. The micrographs are 1- $\mu\text{m}$  thick optical serial sections through the middle of the cell aggregate. The sequential sections are read from top to bottom, left to right. The subcellular location of

microspheres within individual cells in aggregates can be precisely determined from the combined CSFM and DIC images. Note that the yellow microspheres appear three-dimensional because of the superposition of the shaded DIC image on the CSFM image. Full picture width = 25  $\mu\text{m}$ .

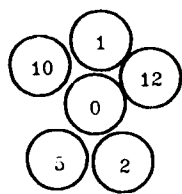


Fig. 5. This diagram shows the relative position and the number of microspheres inside each cell seen in Figure 4. The number of microspheres in each cell was determined from the complete set of twelve serial sections through the aggregate seen in Figure 4. In this aggregate, there are  $5 \pm 5$  microspheres per cell (mean  $\pm$  standard deviation), 0 to 12 microspheres per cell (range), and 5 microspheres attached to the outside of the cells. Combined CSFM and DIC imaging is unique in that it can quantitate phagocytosis in individual cells aggregated together.

CSFM, in combination with DIC and computer image processing, is particularly useful for determining if microspheres are inside or outside of individual cells, for studying phagocytosis of cells aggregated together, and for observing the subcellular fate of ingested particles.

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