

Confocal Microscopy for Diagnostic Cytology

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Introduction

Laser scanning confocal microscopy has been used to analyze cytological specimens for research purposes. However, for many pathologists, its application in diagnostic cytology still is *terra incognita*. In our laboratory, confocal microscopy has been used for seven years, mainly to solve problems in cervical cytology, in breast aspirates, and for endometrium cytology.

In contrast to histology where we can cut new sections from the block, the cytologic sample is often limited to one smear. Accordingly, when we wish to expand our diagnostic possibilities, we have to extract the desired additional information from that single smear. This is possible by first de-staining the original smear and subsequently re-staining it. In this article, we describe how we restain old slides with the fluorescent dyes Ethidium Bromide and Eosin, a DNA/protein stain, resulting in staining patterns comparable to the diagnostic Papanicolaou and Giemsa methods in that there is an optimal distinction between nucleus and cytoplasm. The DNA

of the nucleus (Ethidium Bromide) results in a red fluorescence, the protein in the cytoplasm (Eosin) in a green one.

Methods

Both wet-fixed Papanicolaou-stained, and air-dried Giemsa-stained smears can be used for confocal microscopy. The preexistent stain has to be removed by destaining after removal of the coverslip using xylene immersion. Destaining is achieved by rehydrating the smear to distilled water, then immersing in acid alcohol for 20 minutes, followed by 20 minutes in 1 N HCl. The specimens are rinsed with tap and distilled water.

The procedure for the DNA/Protein stain is as follows:

1. Place the dehydrated smear for 1 min in 1% Eosin Y (in distilled water), Merck, Prod. No. 15935.
2. Rinse in distilled water.
3. Stain for 5 min in 0.01% Ethidium Bromide, Merck, Prod. No. 11615 (pH = 5.7).
4. Rinse in distilled water.
5. Mount the smear in Aquamount, BDH Chemicals, Prod. No. 36086.

All images were originally produced using the BioRad MRC-600 confocal imaging system equipped with a Krypton/Argon-mixed gas laser. The objective used was a 60x planapo NA=1.4 oil, (160-017 Nikon) with the K1 and K2 filter blocks in the scan head. This resulted in the signal from Ethidium Bromide (DNA, red fluorescence) appearing in PMT1 and that for Eosin (protein, green fluorescence) appearing in PMT2. For some images, the signals from PMTs 1 and 2 were merged. For the three-dimensional reconstructions, exclusively PMT1 images were used. The image data were stored on a floppy disk.

Results

First, when we try to look into thick epithelial fragments in the smear by conventional means, out-of-focus flare leads to blurred images, lacking in useful detail. However, using confocal microscopy, 'optical sectioning' with the laser beam, produces a 'thin section' from the material on the glass slide, in a similar way to cutting a paraffin block with a microtome. Consequently, in these otherwise imperfect images we are now able to visualize chromatin patterns in great detail (Fig. 1). In addition, (due to the thin sectioning), we can detect mitotic figures (Fig. 2) and glandular lumina in the central part of the fragment, both of which were invisible without confocal microscopy. In fragments from endometrial samples, we can visualize the transition from cystic hyperplasia to adenocarcinoma (Fig. 1a).

Secondly, we can use confocal microscopy to define the bacterial flora in the cervical smear. Here, the high resolution of the system allows dependable recognition of the shape and form of these small organisms, even allowing quantitative measurements to be made. In these thin sections, we can visualize the interrelationship of microorganisms such as Actinomyces and Entamoeba Gingivalis in the smear (Fig. 1b). In these confocal images, the fine details of the microorganism structure approach a level hitherto

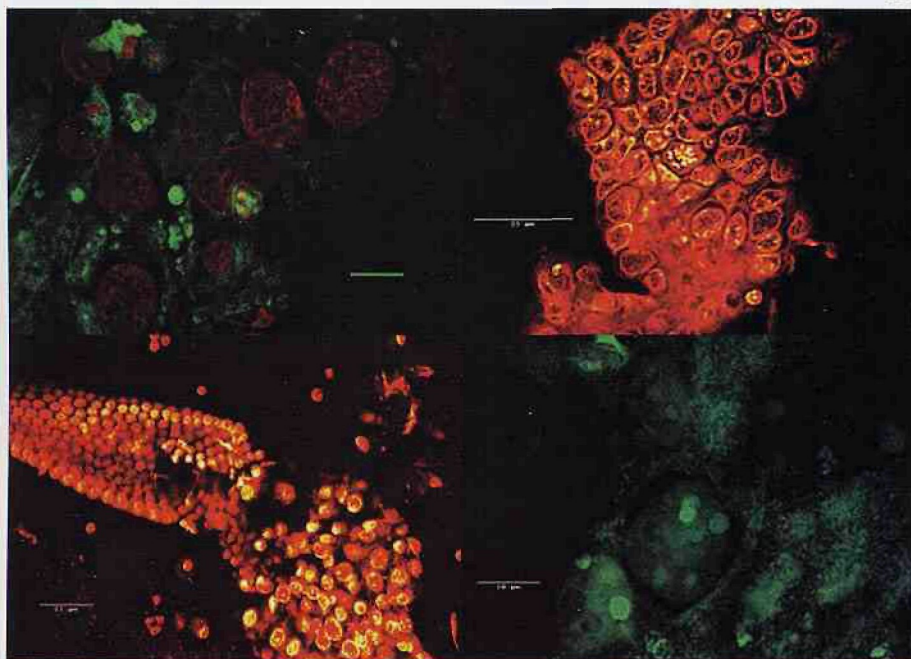


Fig. 1 Optical thin section of a thick epithelial fragment in a cervical smear, merged images of PMT1 and 2. The patient had a poorly differentiated squamous cell carcinoma. In the original Papanicolaou-stained smear, the chromatin was completely blurred. In this confocal image, the fine details of the malignant nuclei are highlighted. Scale bar = 10µm

Fig. 1a Optical thin section of an epithelial fragment in an endometrial smear, PMT1 (DNA). We can observe a direct transition for cystic hyperplasia with small nuclei (left) into frank carcinoma with large nuclei containing prominent nucleoli. Scale bar = 25µm

Fig. 1b Several Entamoeba Gingivales attached to Actinomyces, found in a cervical smear, PMT1 (DNA). This degree of detail of the Actinomyces is not visible in the Papanicolaou-stained smear. The relationship of the two microorganisms is highlighted in this thin confocal section. Scale bar = 25µm

Fig. 1c Three-dimensional reconstruction made from serial confocal sections derived from the microorganism Leptothrix with attached coccoid bacteria in a cervical smear PMT1 (DNA). Here, confocal microscopy approaches scanning electron microscopy in terms of the information it provides. Scale bar = 5µm

unknown to the practising pathologist. In the serial sections, we can see whether the microorganism has actually invaded the cell or is merely sitting on the membrane, (which is impossible to see in the Papanicolaou-stained specimen).

Thirdly, we can gain a great deal of information from the three-dimensional reconstruction of the architecture of microorganisms and cells present in the smear and visualize the surface properties in a similar way to that which we encounter in Scanning Electron Microscopy (Fig. 1c).

Discussion

In cytodiagnosis, analysis of the nuclear pattern is a key issue. The nuclear images in abnormal epithelial fragments, as visualized in confocal microscopy, closely resemble those of plastic-embedded samples. Due to the absence of out-of-focus information, the chromatin patterns seen are less 'coarse' than those observed by conventional light microscopy of the smear. Because of the many cell layers present in these tissue fragments, conventional microscopy produces visualization problems comparable to those present when dealing with thick histological sections (e.g. 30 μm). In this case, superposed cell layers that are more or less out of focus give an undesirable blur to the images of the cell layer that is in the plane of focus. Additionally, the packing of nuclei in the epithelial fragments of smears is relatively dense (particularly when the cytoplasm is immature). This is due to the fact that this cytoplasm has become 'deflated' compared with embedded tissue where it remains in its original shape and size through infiltration by plastic or paraffin. By the use of confocal microscopy, there is no blur in the optical sections obtained, and many of these difficulties are overcome.

For the other examples presented in this article, the benefits of the confocal images are mainly due to higher magnification, serial

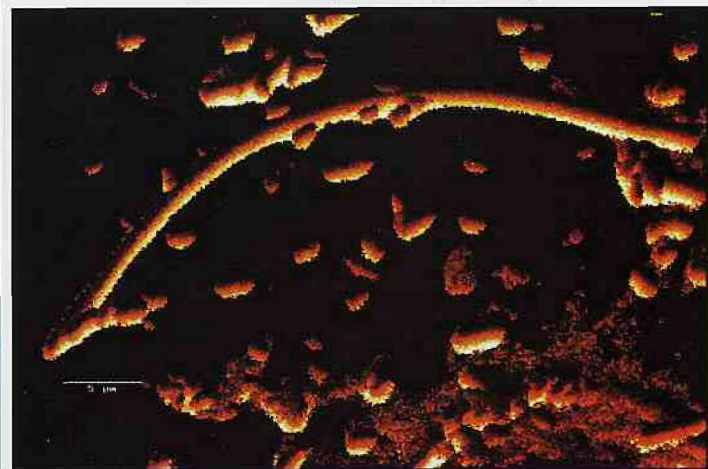


Fig. 2 Optical thin section of an epithelial fragment of a cervical adenocarcinoma in a smear, PMT1 (DNA). In the original Papanicolaou-stained smear, no details or mitotic figures were visible. The fragment looked solid, devoid of a glandular lumen. In this confocal thin section, each nucleus is visualized and a mitotic figure can be discerned. In the lower part of the section a glandular lumen becomes visible (this became larger in the adjacent serial sections), while in the Papanicolaou-stained smear this epithelial fragment was a solid looking object. Scale bar = 5 μm

sectioning, and revealing surface characteristics. In serial sectioning, architectural features can be highlighted, such as glandular, tubular or cribriform built from the tumor tissue. Here, confocal cytology comes close to thin serial sectioning in histology. In displaying surface characteristics of cells and microorganisms in cytologic specimens, the scientific quality of confocal imaging approaches that seen with the scanning electron microscope. Seeing these im-

ages, the cytologist becomes aware that cytology is very much a three-dimensional rather than two-dimensional study.

Conclusion

In the cytology handbooks, one does not find photographs of thick epithelial fragments because they are neither photogenic nor diagnostic due to the inherent blur and lack of detail associated with conventional microscopy. However, by using a simple fluorescent DNA/protein stain in combination with confocal microscopy, valuable diagnostic information can be regained from these important, otherwise undiagnosable, smear elements. In this article, we have restricted ourselves to one simple staining method. However, we have learnt that many more staining techniques can be used. To obtain confocal images, fluorescent probes for proteins and viruses can be applied on de-stained smears, and the proliferating cells can be labeled for confocal microscopy with MiB-1. Finally, the confocal digital images can be processed further, for instance by the sophisticated Application Visualization System (AVS) on a CONVEX computer, leading to even finer details than shown in the illustrations. ■

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