On the Application and Possibilities of In Vivo Microscopy in Liver Research

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ON THE APPLICATION AND POSSIBILITIES OF IN VIVO MICROSCOPY
IN LIVER RESEARCH

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Abstract

In vivo microscopy (IVM) provides a valuable method for studying the histophysiology of the living liver. The method allows observation of living cells in the intact organ of an anesthetized animal with an undisturbed microcirculation, at a magnification and a resolution comparable to normal light microscopy of sectioned material. Due to the absence of preparative procedures, the image differs substantially from histological sections, but it has the advantage of providing us with a reference preparation free of artifacts. In the case of the liver, we have the opportunity to observe directly such details as bile capillaries, intracellular fat droplets, lysosomes, nucleoli and different types of sinusoidal cells and blood cells.

By using epifluorescence, it is possible to visualize the phagocytosis of 0.8 µm fluorescent latex (or other) particles by Kupffer cells, to observe fluorescing substances such as FITC labeled asialofetuin during the process of endocytosis and intracellular transport in parenchymal cells, and to study the behavior of specific cell types such as white blood cells which stain specifically with acridine orange.

It might be expected that in the very near future, the application of modern techniques based on processing TV images, such as image intensifying, averaging, filtering, integrating, gating and others will tremendously improve the possibilities of IVM and bring it to the level of observing and following single molecules, such as FITC labelled peptide hormones and other probes, at the cellular level.

Key Words: In vivo microscopy, liver microcirculation, sinusoid, parenchymal cell, bile capillary, sinusoidal cell, red blood cells, white blood cells, platelets, endothelial cells, Kupffer cells.

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Introduction

Light, transmission, and scanning electron microscopy (LM, TEM, SEM) are widely used as powerful tools to reveal cellular (fine) structure and changes of these structures during various experimental conditions or pathogenesis. In the demand for ever increasing resolution and detail, sophisticated microscopes and preparative methods have been developed which make use of chemical fixation, and dehydration followed by embedding and sectioning for LM and TEM, or by critical point drying and sputter coating for SEM. In case we are aware of looking at artifacts, freezing methods (etching, fracturing) are generally accepted as an alternative to chemical fixation. Methods for the microscopic study of living cells are mostly restricted to cell cultures, using an inverted microscope with a contrasting mode such as phase- or interference contrast. Attempts to look at living cells in a wet chamber in the high voltage TEM have only partly been successful (30).

The purpose of the present paper is to review recent applications of in vivo microscopy (IVM) and to speculate on future possibilities of the method in liver research.

The images obtained by IVM have an excellent illustrative and educational value, and form a valuable complementary visualization technique, in addition to LM, TEM, and SEM. Apart from this illustrative aspect, IVM can be used as a powerful research tool, enabling the investigator to observe living cells in their natural environment, and to study experimental or diseased states of the tissue. By applying the method of epifluorescence, one is able to follow dynamic processes such as endocytosis and subsequent intracellular events. Fluorescing compounds and particles or labeled cells can be visualized with this method. In the last part of this paper we will speculate on future trends and try to define promising developments which will open new possibilities for IVM in liver research.

Materials and Methods

The apparatus

In another paper in this proceedings, a detailed description is given of the equipment necessary for IVM of organs (see the paper by McCuskey and refs 3, 22). Nevertheless, we will

briefly describe the apparatus used by us to study the behavior of white blood cells in sinusoids.

As a light source, a high pressure Xenon lamp is coupled to a monochromator (3, 22), providing monochromatic light in the range of 370 nm to 1100 nm. This offers the possibility to vary the contrast of red blood cells (RBC), which is maximal at 550 nm, and minimal at wave lengths above 600 nm. A long distance condenser transilluminates the margin of the liver, which rests on an optical grade mica window mounted in the special animal stage. Dry or water immersion objectives in the range of 10-100 x provide the possibility of working at different magnifications. A high pressure mercury light source is attached to a Ploemopak® (Leitz) equipped with different filter-combinations to provide epifluorescence. A sensitive TV camera, connected to the phototube of the microscope visualizes images on a TV monitor and offers the possibility to record images on a semi-professional TV recording system, such as Umatic. The application of such an instrument forms an ideal and economic way to record observations which can later be transported, exchanged and filed. Video cassettes also have two audio channels for oral reports or comments. Apart from the TV possibilities, a direct observation of the preparation is possible. At low magnification, this mode of observation is used for orientation and critical evaluation of the liver lobes. Once an area is selected for more detailed studies, the TV mode is used for further observation, because the ability to adjust the contrast and brightness of the TV system usually provides better image definition than obtainable with the human eye. Other possibilities for recording images consist of a photocamera, equipped with an electronic flash as the microscope light source, or a cine camera.

Preparative procedure

Young rats (150 g) are anesthetized and the liver is exposed by laparotomy. It is important not to touch the liver with the fingers or with tools, because this will damage the delicate surface of the tissue and introduce local blood flow disturbances. One large, central lobe of the liver is gently extruded from the peritoneal cavity by exerting slight pressure on the abdomen. The exteriorized liver lobe is made to rest on the mica window of the special microscope stage and covered by a small piece of transparent Saran wrap. The abdomen is wrapped in gauze saturated with Ringer's solution to prevent the intestines from leaving the peritoneal cavity through the incision. In order to arrest respiration movements, the liver also is covered and supported by pieces of wet gauze, except for the area of observation. The lobe is constantly kept wet by flushing it with Ringer's solution at 37 °C through a small tube which is attached to the water immersion objective lens by an elastic band. Ideally, the preparative procedure should be short. Catheters for i.v. injections can be placed at several places in the body to introduce all kinds of solutions or suspensions, such as the one for continued anesthesia with i.v. Nembutal. Anesthesia can be checked by touching the cornea : reactions of the eyelids indicate the need for more anesthetic. Preparations like this can be used for 3 to 4 hours, except when injections are done, which might limit the period of observation.

Observations and Discussion

At lower magnification (10-20 x dry objective lenses), the margin of the liver can be transilluminated, and the larger vessels with their ramifications can be observed. Central veins are seen to approach the outermost margin with rounded, sometimes branching curves. Portal veins are difficult to find, especially in adult rats (9); sometimes they can be seen hidden in deeper layers of the tissue. However, in most livers, regions can be found where both portal and central vein branches are present. One would expect that a detailed study of the functional units of the liver tissue, i.e. the lobule or the acinus (1, 18, 20, 23, 35) should be possible in such areas. However, there are several factors which limit the possibilities of IVM : the local microanatomy in the thin and outermost regions of the liver lobe differs from deeper parts (32), and the superficial sinusoids are located in a plane parallel to the surface. Furthermore, the depth of focus of the LM will restrict the image to a volume of tissue comparable with a histological section. Apart from portal and central veins, sometimes hepatic arterioles and their terminations (1, 20, 35) can be recognized by their fast blood flow. Connections to portal veins (1, 20, 32) and sinusoids can be observed. Bile ducts are usually not observed. Other species, including frogs (11, 12, 20), mice (11, 24, 26), cat (12), hamster (12, 24), guinea pig (10) and dog (12), but mostly young rats or even fetal rats (21) have been used for this type of study.

At higher magnification, excellent images can be obtained by using water immersion lenses (e.g., 40-100 x). Cellular detail is of a quality comparable to an LM derived stained histological section. Apparently, the image details are restricted to the depth of focus of the objective lens, resulting in an optical section. By turning the fine focus control, the microscope provides us with sequential sharp images, which contain valuable 3D information. Amazingly, the images of these water immersion lenses show excellent contrast and detail without the necessity to apply contrasting methods such as phase or interference contrast. The image is not blurred or influenced unfavorably in any way by the presence of thick layers of living tissue at both sides of the focus plane of the objective lens.

Within the liver, different cell types can be observed, such as parenchymal, Kupffer, endothelial, and fat-storing cells as well as different types of blood cells. Parenchymal cells show several details, such as bile canaliculi, fat droplets, nuclei and nucleoli, and several granular organelles, some of which can be suspected by their topography to be lysosomes and mitochondria. Unfortunately, the space of Disse at the sinusoidal border of the cell, and the thin layer of endothelium covering this space, cannot be separately distinguished. The perikaryon of endothelial cells can be recognized by their streamlined appearance. Fat-storing cells can

have comparable morphology, but they differ due to the presence of fat droplets. Kupffer cells have an irregular shape, and can be unequivocally recognized after the phagocytosis of a small number of (fluorescent) 0.8-1.0 μm latex particles. The perinuclear region of endothelial cells and Kupffer cells can bulge into the lumen and influence the passage of blood cells (20, 25, 45). Since these cells possess a mechanism of contractility, they may act as local sphincters (1, 12, 20, 41) influencing the distribution of blood through the tissue. Pit cells have yet to be identified since they presently cannot be discriminated from lymphocytes and other white blood cells which are present in the sinusoids. RBC and white blood cells (WBC) can be studied in detail and their interaction with the sinusoidal wall will be reported elsewhere. WBC can be recognized from the presence of granules, the shape of the nucleus and the diameter of the cell, but precise recognition is not always possible. RBC velocity in sinusoids has been measured and maximum values amount to 400-500 μm/sec (5, 28). RBC are extremely deformable during their sinusoidal passage. WBC are much more rigid (20, 45) and they periodically obstruct the sinusoid due to their larger diameter (43). Blood platelets can be frequently seen, and their behaviour studied particularly in sinusoids having reduced or zero flow.

The method of IVM also allows a detailed study of sinusoids. Sinusoids are recognized by the presence of moving RBC. The average sinusoidal diameter is reported to be 6.3 μm (45). Periportal and central sinusoids were found to differ in diameter from 5.9 to 7.1 μm (45) and in tortuosity (23, 43). These observations can be confirmed by IVM, periportal sinusoids are narrower than central ones (45). This observation is of importance because it means that the wall of periportal sinusoids will interact to a greater extent with blood cells, resulting in processes like forced sieving (45) and endothelial massage (45). Other observations concern the parallelism of sinusoids, which is more pronounced in centrolobular areas (23, 43) : the sinusoidal hematocrit, which can vary largely : the irregularity and sometimes reversal of periportal blood flow : and the occurrence of bars in the sinusoidal outlets in the central vein (43). Some authors report that the influence of respiratory movements result in intermittent flow in the sinusoids (7, 31, 34, 41), but we do not observe this under normal conditions.

The use of 80-100 x water immersion lenses has implications for the study of liver tissue. Due to the short working distance, the surface of the preparation is sometimes touched and compression can limit or stop blood flow. The small depth of focus, together with the small working distances limits the observation to the superficial 2-3 cell layers (i.e. 40-60 μm) of the tissue. With transillumination methods, the zone of observation is limited to a few millimeters at the margin, but with epi-illumination methods much larger areas of the liver can be investigated.

With the aid of 0.9-1.0 μm fluorescent latex particles (24-27), the specific uptake by Kupffer cells can be observed. Fluorescent molecules, particles, liposomes and other carriers, can be visualized by using the epifluorescent mode of the microscope. This allows specific localization of the cells which take up the material; it further allows the time lapse study of the route of entrance into the cell, i.e. the mechanism of endocytosis (phagocytosis vs pinocytosis). The number of biological macromolecules which are known to be taken up by specific receptor mediated uptake is rapidly increasing. Receptor mediated uptake is often specifically located in one particular cell type. FITC labeling of the molecule concerned, results in direct visualization of the particular cell, together with a visualization of the route of uptake and the fate in the cell. It might be expected that these substances, together with vital stains, cell type specific antibodies, and other specific targeting techniques, will provide us with the means to recognize unequivocally cells in IVM in the liver.

Due to a gift by Drs. J.W. Slot and J.J. Geuze, we were able to visualize the uptake and lysosomal accumulation of FITC labeled asialofetuin in living parenchymal cell lysosomes using a very sensitive television camera. After injection of 400 μg of the labeled material in a 350 g rat, we were able to visualize the fluorescence in granules adjacent to bile capillaries. It might be expected, that by further refinement of the method, the uptake and processing of such substances in CURL, the compartment of uncoupling of receptor and ligand (6), could be visualized. With fluorescent substances one might expect to approach, or even surpass, the limit of resolution of the LM, due to the "dark field effect" i.e. the visualization of incoherently emitting sources below the usual resolution limit. In fact, IVM has a great potential in the study of the in vivo behaviour of labeled molecules, particles or other carriers in relation to cellular distribution, endocytotic mechanisms and intracellular route and fate.

IVM in experiments and disease

A large number of experimental conditions and animal models of human diseases has been studied with IVM. It is not the purpose of this paper to review these experiments and to draw conclusions. However, in order to illustrate the capacity of IVM for liver research, we mention the use of IVM in the study of :
- alcohol (34)
- endotoxin (24-26)
- toxic compounds, such as CCl4 (17, 19, 28), and thioacetamide (40)
- haemorrhagic (8, 12, 16, 39), traumatic (8) or cardiogenic shock (13)
- murine hepatitis (4)
- Schistosomiasis infection (2)
- veno-occlusive disease (7), portal hypertension (34)
- portacaval shunt (27).

This list is not complete, but it demonstrates the applicability of IVM in experimental pathology, especially with regard to changes in flow and/or microcirculation.

Apart from the above mentioned experimental situations, the majority of studies have been directed to the influence of vasoactive substances and related treatment on liver microcirculation (5, 8, 14, 15, 20, 28, 29, 32, 36, 37, 38). A number of adrenergic (15, 20, 36), cholinergic

(14, 20, 37), and aminergic substances (38) have been tested, together with antagonists or blockers, to determine the effect on sinusoidal diameter, RBC velocity (5) and volume flow (19). Also, the effects of vagal stimulation (14, 36) has been studied. These treatments can either increase or decrease the size of the sinusoids, indicating the presence of contractile mechanisms in the sinusoidal lining cells. The exact mechanism of action of these vasoactive substances and the nature of the possible contractile cytoskeleton of the sinusoidal cells is at present unknown.

New developments, new equipment

So far, we may conclude that IVM has contributed in the field of liver research, but due to the availability of improved TV equipment and computer-based processing techniques, the possibilities for new approaches are offered and form a scientific, technical and, unfortunately, financial challenge.

It is possible to enhance the sensitivity of the TV system dramatically by using an image intensifier to lower the threshold of detectability to the level of, probably, single photons. This means that very low doses of FITC labeled substances can be followed at the cellular level.

New promising TV techniques are the application of averaging, filtering, stretching and computer-processing of images. In some instances, we do not need the movement and the 50 or 60 images per second, and two or more images might then be averaged. This enhances the signal to noise ratio and the image quality considerably. Once the image is in a memory, we can also process by applying techniques such as densitometry, (semi)automatic morphometry, etc.

The application of more sensitive color camera or color systems allows working with natural, introduced, or artificial colors. With transilluminated white light the presence of natural pigments, introduced vital stains or different fluorescent dyes can be visualized. Artificial color techniques or pseudo color can be used for signals obtained from two different images or two or three different wavelengths and depicting it as an artificial, colored image.

Further possibilities for liver research

The study of the uptake of labeled compounds or small objects will tremendously be facilitated by the new improvements and developments of digital video techniques. It is supposed that the combination of image intensifying and image enhancing (averaging) techniques will possibly lower the threshold of detection to the level of single molecules. If this is true, we might be able to visualize the arrival, uptake and cellular processing of single molecules, such as drugs or hormones, in cells in the living tissue. Also, these visualization techniques will enable investigators to follow the uptake of artificial particles (microspheres, liposomes, immunogold) or natural particles (chylomicrons, remnants and other lipoproteins) and study the cellular distribution and endocytotic mechanisms concerned (33). Targeting of probes to different cell types can be observed directly by IVM and does not need complicated techniques of cell preparation and further measurements.

Not only molecules and particles can be visualized, but also the introduction of cell suspensions can be followed by IVM. The introduction of metastasing cell suspensions can be followed by IVM. The introduction of metastasing cells, or transplanted cells can be followed in the liver in situ.

Further possibilities are observations on the effects of perfusing a fixative, or other solutions through the liver and see what the effects are (42). If such experiments are combined with in vivo morphometry, valuable data are obtained for comparison with morphometry based on sections or SEM preparations (44).

Furthermore, one might consider the possibilities of applying IVM to the human liver. This seems to be difficult, due to the presence of a thick collagenous (Glisson's) capsule covering the organ. If this capsule could be reduced in thickness and if modern endoscopic techniques could be brought to the microscopic level, then there exists a chance to visualize a small part of the liver microcirculation.

The further development of in vivo liver morphometry should be encouraged, since 3D reconstructions of cells and sinusoidal networks seems to be possible by using the images of different levels of focus.

The use of phase and interference contrast should be investigated further; the possibilities of these techniques in epi-illumination modes seems to offer new possibilities for the visualization of even more detail at the organelle level.

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