Food Structure

Volume 6 | Number 2 Article 2

1987

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FOOD MICROSTRUCTURE, Vol. 6 (1987), pp. 115-120
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0730-5419/87$3.00+.00

CONFOCAL SCANNING LASER MICROSCOPY IN FOOD RESEARCH: SOME OBSERVATIONS

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Abstract

Confocal Scanning Laser Microscopy (CSLM) has advantages over conventional light microscopy and electron microscopy. In particular the possibility to perform optical sectioning, allowing the disturbance free observation of the three-dimensional internal structure, offers new possibilities in microstructural studies of food systems. The technique is further considered to be very valuable in the study of dynamic processes and transport phenomena. Preliminary results are discussed of an investigation into the microstructure of fat spreads, mayonnaise, cheese and rising dough using this CSLM technique.

Initial paper received October 8, 1987
Manuscript received October 30, 1987
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Key words: Light microscopy, confocal microscopy, laser, three-dimensional imaging, food microstructure, margarine, butter, mayonnaise, cheese, dough, dynamic processes.

Introduction

Light and electron microscopy play an important role in the observation of the microstructure of food systems. Electron microscopy (EM) has the advantage of a high resolution (~1 nm), but it is generally very laborious and requires elaborate sample preparation which may lead to artifacts. In addition, samples are observed under high vacuum and at high radiation doses. Classical light microscopy (LM) is easy to apply but its resolution is rather limited and undisturbed observation of samples is difficult, especially when deeper layers must be probed.

In this context, and in view of the desirability to perform measurements under dynamic conditions, alternative techniques such as acoustic microscopy, X-ray microscopy and confocal scanning laser microscopy (CSLM) may be considered.

The CSLM technique has already been discussed extensively (1, 2, 6, 8, 9). The basic idea of the confocal principle is that a point in the object is optimally illuminated and also optimally imaged in a detector pinhole, which leads to an increased resolution and a reduced depth of field because off-focus levels in the specimen will hardly contribute to the image. The latter property enables an excellent optical sectioning (i.e., the in-depth imaging of the structure) and a disturbance-free three-dimensional observation of a non-deformed sample, which cannot be achieved with classical LM. In addition, computer control and data acquisition create excellent possibilities for image analysis and processing.

Preferably, specimens are studied by fluorescent light because the different structure elements can best be distinguished by using specific fluorochromes. Also in this case, the point illumination and pinhole detection system is a great advantage of CSLM over conventional LM because it effectively suppresses contributions from off-focus levels of the object.

The aim of the present contribution is to present some of our first results on the observation of food systems by CSLM.

Experimental

General

Samples were observed by the CSLM developed by the Department of Electron Microscopy and Molecular Cytology, University of Amsterdam. Observations were made with oil-immersion optics (N.A. = 1.3) in the reflection / fluorescence mode (1, 2, 8), allowing visualization of structures to a depth of

more than 100 μm under the surface of thick, non-deformed specimens.

Fat spreads

The water distribution in spreads containing 80% fat (commercial margarine and butter) was investigated. Samples of the product were taken by means of a sampling tube (Fig. 1) fitted with a sharp edge to prevent working of the sample as much as possible. When the tube was full, a stopper was inserted on the other end and the sample pushed out. The material protruding from the tube was cut into slices 2 mm thick by means of a thin platinum thread (0.1 mm).

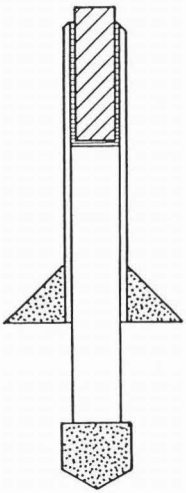


Fig. 1. Schematic drawing of sampling tube for taking specimens of fat spreads. Material: stainless steel; total length 80 mm; internal diameter 5 mm.

Two or three of such slices (diameter = 3 mm) were placed on a cover glass, which was introduced in to a container containing a fluorescent dye. For a proper observation of the water globules in a fat spread, a fluorescer has to be added either to the oil or the water phase. Nile Blue is a suitable dye for the localization of lipids by fluorescence light microscopy (3, 10). The samples of fat spreads were immersed into an aqueous solution of Nile Blue (0.001 % w/v) for at least 16 hours. This solution contains a number of lipophilic components, which diffuse into the liquid lipid phase, thus generating a deep yellow fluorescence. The lipid phase becomes fluorescent without altering the structure of the emulsion.

Mayonnaise

In order to maintain the structural integrity of the original mayonnaise as much as possible, nine parts of a mayonnaise sample were carefully mixed with one part of a Nile Blue solution (0.1 %). In contrast to fat spread samples, the components in the continuous water phase as well as in the interface become fluorescent (or at least more fluorescent than the discontinuous dispersed fat phase). In this case, the fluorescence of the hydrophilic components of the Nile Blue dominated that of the lipophilic components, which might be attributed to hindered transport. After staining, the mayonnaise sample was placed between two glass slides.

Cheese

A small piece of young Gouda cheese was placed on a glass slide and introduced in a 0.01 % Nile Blue solution to stain the fat phase or, alternatively, in 0.01 % solution of 1-anilino-8-naphthalene sulfonic acid (ANS) to stain the protein.

Rising Dough

To establish the capabilities of confocal instrumentation for analyzing dynamic processes in situ, the structural changes in rising dough were studied. To this end, 10 g of wheat flour was mixed with 5 ml of an 0.05 % fluorescein isothiocyanate solution in water and 0.5 g of yeast. After kneading for 10 min and a first rise for 30 min, a small piece of dough (thickness 2 mm) was placed between two glass slides and observed at room temperature (20°C) as a function of time.

Results and Discussion

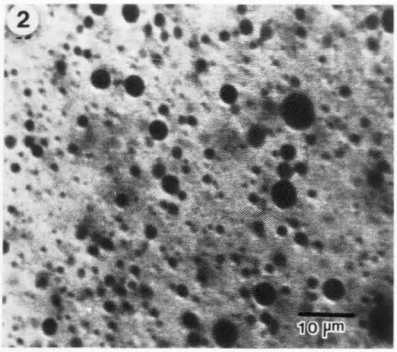
Figs. 2 and 3 show the water distribution in margarine and butter samples, respectively. The water droplets appear as dark non-fluorescent structures embedded in a fluorescent fat phase. Because of the high resolution and high dynamic range, structures as small as 0.3 μm can be discerned without difficulty. This cannot be achieved by normal light microscopy, although special imaging modes may come very close (7). The main advantage of CSLM is that pictures can be taken at different depths without deformation of the samples. This possibility to make optical sections along the optical axis is demonstrated in Fig. 4 for a mayonnaise sample.

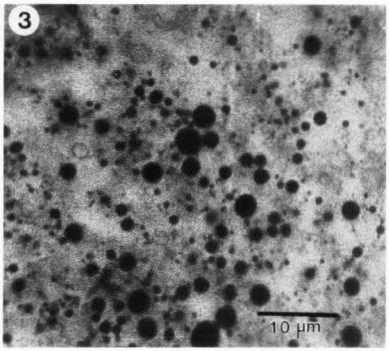
Mayonnaise is an oil-in-water emulsion containing a high percentage of oil (80 % or more). This high volume of oil causes the formation of a honeycomb structure of closely packed and often distorted oil droplets (the closest packing of equal spheres would lead to a volume fraction of 0.72). The interface layers between the droplets are imaged with a dimension of 0.2 μm, which is very close to the theoretical resolution limit of the instrument.

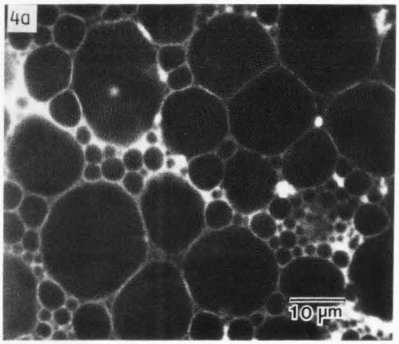
The depth resolution of the optical sectioning is about 0.7 μm (2), so that elements which are that distance apart, are imaged completely independently of each other and do not interfere with adjacent image planes. Consequently, large oil droplets (size ~ 10 μm) are detected on different image planes, whereas small droplets (~ 1 μm) appear or disappear from the image (Fig. 4).

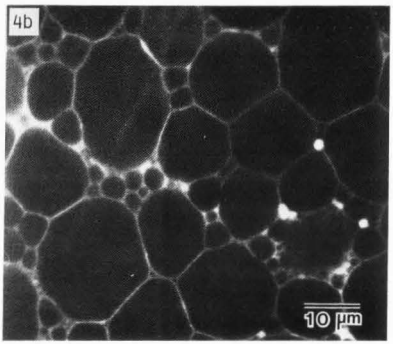
Figs. 2 and 3. Water distribution in margarine **(Fig. 2)** and butter **(Fig. 3)**. Fat phase is fluorescent.

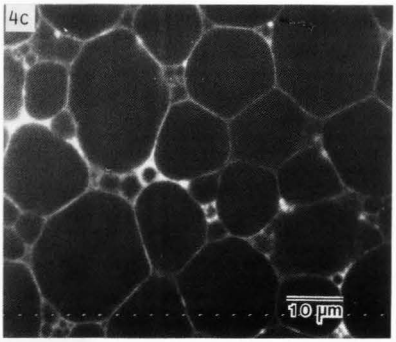
Fig. 4. Mayonnaise obtained at (a) 2, (b) 4, (c) 6, and (d) 8 μm depth below the surface of the sample.

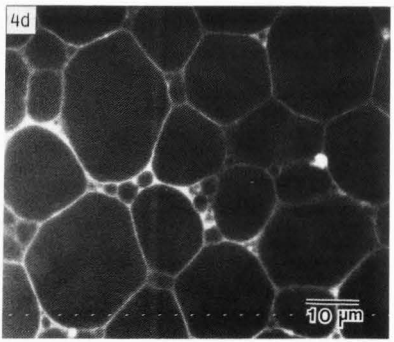

2
10 µm


3
10 µm


4a
10 µm


4b
10 µm


4c
10 µm


4d
10 µm

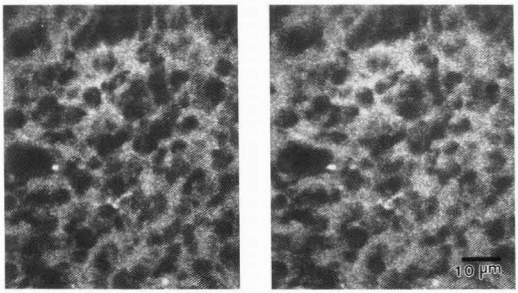


Fig. 5. Gouda cheese. Protein is stained. Stereo-micrographs.

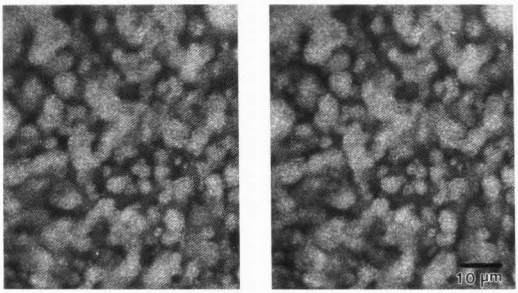


Fig. 6. Gouda cheese. Fat is stained. Stereo-micrographs.

Depending on the optical density of the sample, optical sectioning can be applied up to depths of about 100 µm from the surface (2). A set of images thus formed at specific depths in the specimen can be used to obtain a stereopair of images. A three-dimensional observation of the microstructure requires the storage of data from such a set of images and the generation of stereoscopic images by a specific image processing algorithm (2, 9). Examples of such stereoscopic images are shown in Fig. 5 and 6. Microstructural studies of cheese by LM and EM have been the subject of numerous papers (11). The structural matrix in cheese is protein in which the fat is embedded as a dispersed phase (4, 5, 11). The three-dimensional structures observed by us both with protein staining (Fig. 5) and fat staining (Fig. 6), perfectly agrees with these earlier observations.

The spatial network of protein strands as well as the presence of dispersed fat globules and agglomerates are clearly observable and give an impressive image of the internal structure of the cheese. As already mentioned, this image is obtained without disruption of structure and laborious sample preparation. Moreover, the whole operation takes only a few minutes. It is these performance aspects which make the confocal equipment well-suited to perform in situ observations under dynamic conditions: structure formation in different types of gels, formation of emulsions, coalescence phenomena, and effects of deformation. An example is the study on the kinetics of rising dough, which are related to dough rheology and baking performance.

When mixing flour and water, starch and proteins are hydrated and form a visco-elastic matrix. This matrix can occlude air bubbles. In the rising stage, carbon dioxide produced by yeast, diffuses to the air cells occluded during mixing, thus causing expansion of the dough. The four micrographs (Fig. 7a - 7d), obtained 1, 2, 4, and 6 minutes after the first dough rise, show the results of monitoring this process by CSLM about 20 µm under the surface of the sample. The protein mass and the starch granules generate strong and faint fluorescence respectively. Gas cells are discernable as dark structures in the protein matrix. Expansion and coalescence of gas cells responsible for dough rise, can also be clearly observed. It is possible to do these time-resolved observations on the internal dough structure without any perceptible photo-bleaching of fluorescer taking place.

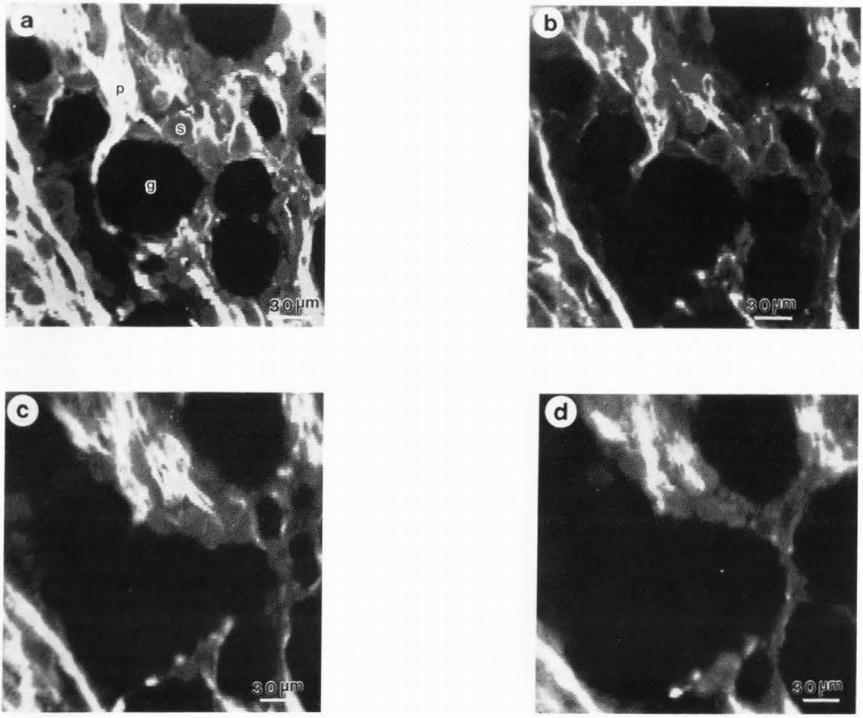


Fig. 7 Rising dough. (a) 1, (b) 2, (c) 4, and (d) 6 minutes after the first dough rise. (p) protein; (s) starch; (g) gas cell.

Concluding Remarks

Confocal Scanning Laser Microscopy appears to be a very useful tool in the study of food microstructure. An improved resolution and a high dynamic range are no doubt advantages over conventional light microscopy. In particular, the possibility of optical sectioning, which allows a disturbance-free observation of the three-dimensional internal structure, offers new possibilities to do microstructural studies of food systems. In addition, CSLM enables the monitoring of dynamic processes as a function of variables such as temperature, pH, concentration, pressure, etc. In this context, the possibility to study transport phenomena by fluorochromes or-gold-labeled compounds - should also be mentioned. These options may mark a new era in the study of food products.

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Discussion with Reviewers

S.H. Cohen: What are the advantages and disadvantages of stage scanning as compared to laser scanning?
Authors: Advantages and disadvantages of on axis stage scanning versus off axis laser scanning have been discussed by Brakenhoff (2) and Petran et al., (6). An important aspect in favor of on axis stage scanning is the somewhat higher attainable resolution. On the other hand, a scanning stage can be a nuisance when manipulation of specimen is required.

S.H. Cohen: What is the nature of the staining agent Nile Blue?
Authors: Nile Blue is a commercial preparation, containing protonated oxazine base, oxazone and free oxazine base.

D.P. Dylewski: I assume that all samples examined in this paper were studied at room temperature. Is it technically feasible to apply a hot or cold stage to the CSLM to enhance its versatility in studying dynamic processes?
Authors: Just as in normal light microscopy it is possible to use hot or cold stages. However, in case of stage scanning, dynamic experiments or micromanipulation can be troublesome.

J.D. Fairing: In Fig. 2 many of the water droplets appear elliptical rather than circular. Is this an optical effect or is it due to the working of the sample?
Authors: We are not quite sure about the cause of this distortion. Most likely it is due to the working of the sample. On the other hand, it cannot be excluded that the scan speeds in both directions are not completely matched.

J.D. Fairing: What is the cause of the difference in appearance of the water-fat interface in Fig. 3 as compared with Fig. 2?
Authors: We cannot offer an explanation for this difference.

J.D. Fairing: In Figs. 5 and 6, what is the equivalent parallax angle of the stereopair?
Authors: The angle is ten degrees.

J.D. Fairing: In Fig. 7, why are the individual pixels so clearly visible when they are not seen in the other micrographs? What is the difference in the instrumental and recording conditions that produces this undesirable effect?
Authors: This effect is caused by the image format of 256-256 pixels, used in producing these pictures. These conditions were applied because rapid recording, of the dynamic behavior of dough rise, was required. Normally, a format of 512-512 pixels, and for high resolution images 1024-1024 pixels, is used.

J.D. Fairing: What is the wavelength of the exciting radiation in your experiments?
Authors: The wavelength of the exciting radiation is 512 nm.