

# Chapter 2

## Tools for the Study of Nanostructures

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### 2.1 Introduction

The design of food materials with improved quality characteristics and beneficial effects for humankind is one of the current trends of food industry (Aguilera et al. 2003 and Aguilera 2005) and is in this context that the scientific community have started to design food items with specific properties, and where the structure at micro- and nanoscopic levels has become very important as it plays a crucial role in food functionality (Aguilera and Stanley 1999). Regarding this subject, many of the currently carried out studies have been done using high-resolution microscopy, and spectroscopy techniques, providing basic and fundamental knowledge about structure, and their relation with processing and functionality.

In this chapter, some of the most used microscopy techniques for analyzing the structure of biological materials at the micro- and nanoscale will be presented. These techniques are usually applied looking for the changes that promote the arise of new properties and phenomena that emerge at these scales, hence, the purpose of this chapter is to provide the reader with examples of the application of different types of microscopy techniques usually used to characterize food items.

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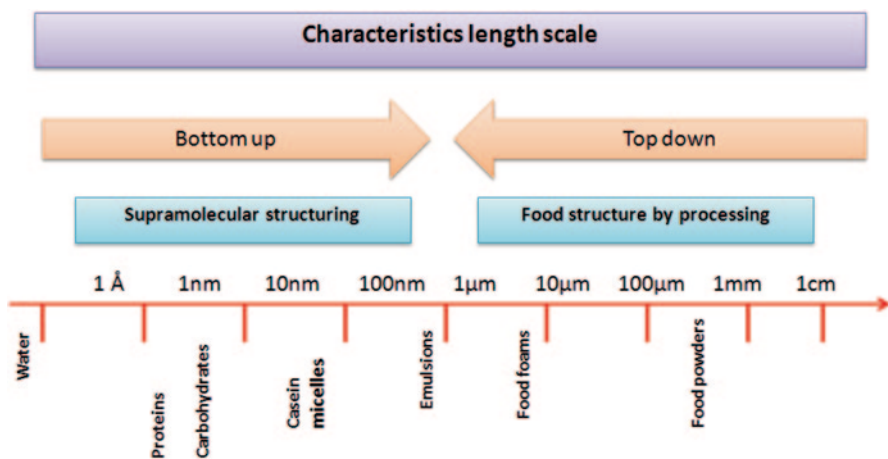
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## 2.2 Structure

Food structure is defined as the organization of food constituents at multiple spatial scales that result in the common food properties, mainly textural. Depending on how the product is studied, it can be catalogued in different ways. At one extreme, a food product can be considered at a macroscopic level, where the whole product is evaluated, for example, to obtain some physical or morphometric characteristics, while at the other extreme, when analyzing its molecular composition it will be categorized at a micro- or nanoscale (Ubbink et al 2008). However, this evaluation will depend on the products' constituents and on the dominant length scales that establish the food properties (Fig. 2.1) and it is in this context that microscopy analysis becomes irreplaceable (Aguilera et al. 2003).

The selection of the microscopy techniques to be used, is based on the elements that composed the structure of the sample and that are wanted to be analyzed (Fig. 2.2) and considering the relevant scale as the dimensional level at which the effects of certain phenomena can be observed or explained. A first distinction is required between macrostructure (bulk or macroscopic level) and microstructure (Aguilera 2000). Nowadays structures can be resolved to the atomic level (nano-structure) but the challenge is to identify the relevant scales responsible for the desired functionality, which may vary from molecules at interfaces to visible particles (Aguilera 2000) and decide which microscopy tools can be used to fulfill this purpose. Table 2.1 shows the main characteristics and the resolution range of the different microscopy systems.



**Fig. 2.1** Characteristic length scales in food and examples of representative food ingredients and food structures

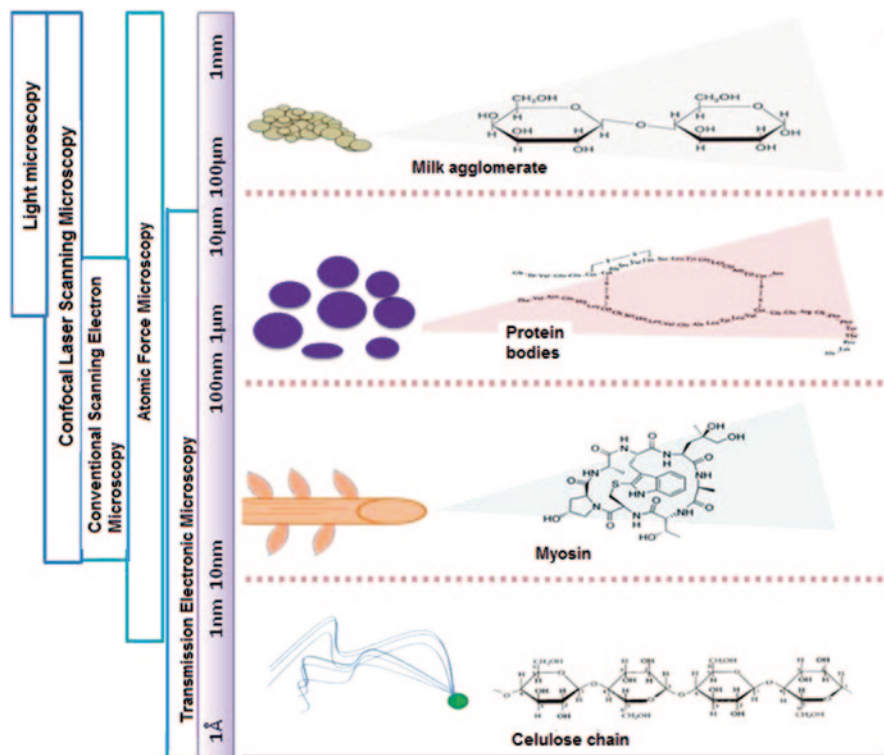


Fig. 2.2 Microscope scales and structural levels used in food science

## 2.3 Tools for the Study of Micro- and Nanomaterials

### 2.3.1 Light Microscopy

Optical or light microscopy applied to food items was initially used in order to detect the presence of contaminants, whether accidental or deliberate. This kind of microscopy involves the pass of visible light, either transmitted through or reflected from the sample, through a group of lenses, resulting in the image magnification of the observed samples (Abramowitz and Davidson 2007). Sometimes the specimens under observation, due to their own natural structure, do not need any special treatment to be observed (amplitude specimens), however, others show so little difference in intensity and/or color that their feature details are extremely difficult to discern, requiring the samples a special treatment or a contrast method such as dark field, fluorescence, or confocal microscopy, but even when applying these methods, the resolution that is possible to reach by optical microscopy is low (0.2 μm) giving rise to some modifications.

**Table 2.1** Features of microscopy systems used in food science and nanotechnology

Microscopy	Resolution	Uses
Photonic	80 nm–5 cm	Amplitude specimens
Stereo	100 $\mu\text{m}$ –5 cm	Thick or opaque tissues. Overall structure from animal or plant tissues
Fluorescence (FM)	120 nm–1 mm	Fluorescence specimens (natural or added with fluorochromes)
Confocal laser scanning (CLSM)	100 nm–1 mm	Enables the reconstruction of three-dimensional structures from images. To study overall structure and changes during processing. Protein and lipid bodies, components differentiation
Multiphotonic	80 nm–1 mm	Subcellular fluorescence and deep analysis in thick tissue. Ultrastructure and nanometric elements can be observed
Scanning electron microscopy (SEM)	3 nm–500 $\mu\text{m}$	Superficial or external structural characteristics. Membranes, myosin, cells, particles, and other cellular structures can be studied
Environmental scanning electron (ESEM)	3 nm–500 $\mu\text{m}$	Low-vacuum microscope for hydrated samples; it is ideal for biological samples
Transmission electron microscopy (TEM)	0.1 Å–1 nm	It is used in biological science for ultrastructural studies to organelles, cell walls, molecular structure of proteins, lipids, and polysaccharides
Scanning probe (SPM) or atomic force (AFM)	0.1–500 nm	Analytic three-dimensional surface profiles can be observed at nanolevel

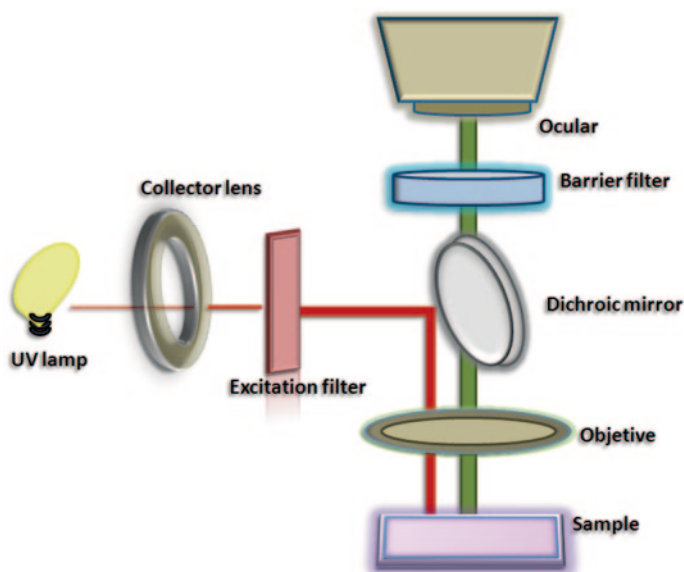


Fig. 2.3 Dark-field microscopy light path

### 2.3.1.1 Dark-Field Microscopy

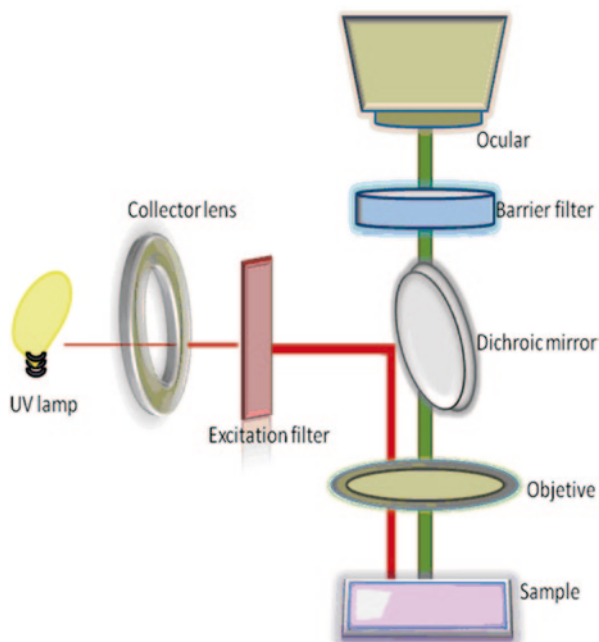
Dark-field microscopy creates contrast in unstained transparent specimens such as living cells. This depends on controlling the illumination of the specimen so that the central light that normally passes through and around the specimen is blocked, allowing only oblique rays to illuminate the observed sample (Fig. 2.3). This kind of microscopy has been used to detect metal nanoparticles (Raschke et al 2003) for biomolecular recognition. Kim et al. (2007) demonstrated the usage of dark-field detection with a rather large bead ( $>2\ \mu\text{m}$ ) for stretching DNA or RNA detecting enzymatic activity. The scattering from metal beads was so intense that it could even be used for high speed measurements as was shown for studying the flagella rotary motor at frequencies as high as 300 Hz.

### 2.3.1.2 Fluorescence Microscopy

Fluorescence microscopy is based on the same common principles applied to optical microscopy (Fig. 2.4), differing in the management related to the generation and transmission of the wavelengths suitable to the fluorochromes to be visualized. Excitation processes usually require short wavelengths in the near UV (quartz, halogen, mercury, etc.).

The basis of the technique lies on the fluorescence properties of some elements or compounds. Usually, fluorochromes are added knowing that they will be bound to the antibodies by a stable chemical bond that cannot be broken during the course

**Fig. 2.4** Fluorescence microscopy



of the immunological reaction. Some compounds have natural fluorescence such as some vitamins, steroids, porphyrins, etc. Other fluorescent compounds such as rhodamine, auramine, fluorescein, and acridine orange are used in various staining techniques.

Fluorescence microscopy methods have been developed with improved optical interference filters, dichroic mirrors, epi-illuminators, sensitive films, and electronic imaging devices. For the application of the fluorescence detection systems, four elements must be compatible: an excitation source, a fluorophore, a filter of a particular wavelength that isolates photon excitation, and a detector that registers the emitted photons and produces a recordable output, usually as an electrical signal or a photographic image (Perucho-Lozano 2011).

Fluorescence microscopy has been used in various fields of microbiology, genetic engineering and physiology, and for observing previously invisible processes, including the development of neurons, how cancer cells are disseminated, the development of Alzheimer's disease, the growth of pathogenic bacteria, and the proliferation of the AIDS virus among others (Pérez-Millán and Becú-Villalobos 2009)

### 2.3.1.3 Confocal Laser Scanning Microscopy (CLSM)

The confocal laser scanning microscopy (CLSM) is a novel technique widely used to characterize the microstructure of the materials related to medical, biological,

and materials sciences. It can be applied to gather the microstructure by a dynamic and nearly noninvasive observation (Dürrenberger et al. 2001; Jekle and Becker 2011).

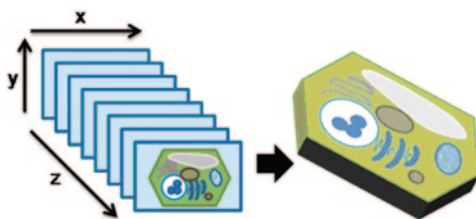
The confocal laser scanning microscope was first conceived by Minsky in 1955, in order to observe individual nerve cells within a packed central nervous system. He designed a simple instrument in which a pinhole was placed in front of an objective and a condensing lens, promoting by this way effectively discriminated out-of-focus light contributions from the specimen (Pygall et al. 2007).

The CLSM proved to be particularly valuable in improving fluorescence images. The optics reject light from outside the focal plane (out-of-focus flare) resulting in images superior in contrast and clarity and with an improvement in the lateral ( $x, y$ ) resolution over standard optical microscopes. The CLSM image is also a true optical section and this capability allows imaging at depth inside translucent specimens. The slice thickness can be as thin as  $0.5\text{--}1.5\text{ }\mu\text{m}$  but is dependent upon the numerical aperture of the objective lens (Pygall et al. 2007).

CLSM is one of a suite of fluorescence imaging instruments, which include: scanning confocal, spinning disc, multiphoton, and wide-field microscope. This equipment has a light source with a scanning unit and an aperture or pinhole (the information from the focal plane), which improves the depth of focus limit (Pygall et al. 2007). The confocal system operates by obtaining information from optical sections focus only on one focal plane, where the sample is excited by the impingement of a specific wavelength laser beam (transmitted or reflected), and where the sample will tend to fluoresce (naturally or induced) (Aguilera and Stanley 1999; Aguilera 2000). This optical section will contain information from one focal plane only, therefore, by moving the focal plane of the instrument by steps of defined distance (range in  $\mu\text{m}$ ) through the depth of the specimen, a stack of optical sections will be recorded. This property of CLSM is important for solving three-dimensional (3D) microstructures (Fig. 2.5), where the information from regions distant from the plane of focus can blur the image of such object (Dürrenberger et al. 2001; Pygall et al. 2007; Perea et al. 2010, Aguilera and Stanley 1999; Amos and White 2003; Achir et al. 2010; Chassagne-Berces et al. 2009).

The CLSM incorporates two operation modes; the first is by illuminating the sample point-by-point and rejecting the out-of-focus light. The principle is shown in Fig. 2.6, where the excitation laser beam (intense blue excitation light) reflects off the light in a dichroic mirror, directing it to an assembly of vertically ( $x$ ) and horizontally ( $y$ ) scanning mirrors; these motor-driven mirrors scan the laser across

**Fig. 2.5** Optical sections of vegetable cell from focal planes ( $x, y$ ) at different depths ( $z$ ) through a cell, which provides a three-dimensional image



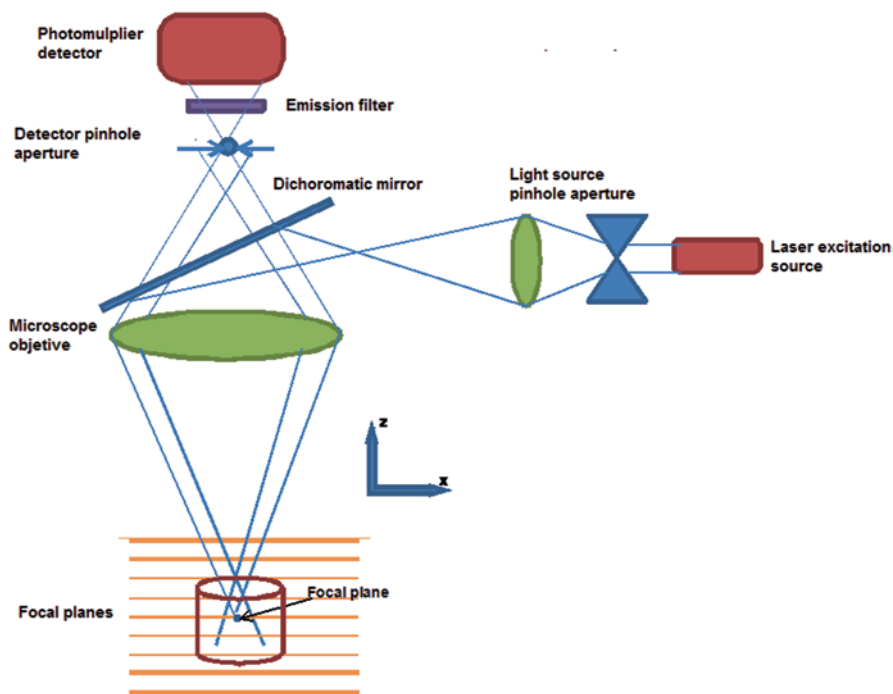


Fig. 2.6 The confocal principle

the specimen through the microscope objective. The fluorescence light emitted by the dye molecules (induced or autofluorescence) passes back through the objective and is descanned by the same mirror through a pinhole placed in the conjugated focal (called confocal) plane of the same; the pinhole thus rejects all out-of-focus light arriving from the sample and the light only from the focal point (plane) can pass the pinhole and be measured. Finally, the emitted light is measured by a detector such as a photomultiplier tube. Then, a computer reconstructs the two-dimensional image plane one pixel at a time (single optical sections), and a 3D reconstruction of the sample can be performed by combining a series of such slices at different depths. This kind of microscopy has been widely used in real time biological experiments applied to living cells and tissue sequences (Prasad et al. 2007; Amos and White 2003; Cox 2002; Semwogerere and Weeks 2005; Dürrenberger et al. 2001; Paddock 2000).

The advantages of the CLSM is the capability to obtain optical sections of different focal planes, providing a larger resolution in a section plane ( $x, y$ ), and a good resolution between the section plane ( $>0.25 \mu\text{m}$  in  $z$ -direction).



## CLSM Applications

The application of CLSM to study the microstructural characterization of food materials has been particularly fruitful in the area of lipid components because optical sectioning overcomes the tendency of fats to smear and migrate. Lipids are also well suited to fluorescent staining. The possibility of combining CLSM with rheological measurements, light scattering, and other physical analytical techniques in the same experiments with specially designed stages offers the opportunity to obtain detailed structural information of complex food systems. Different examples of CLSM application are shown in Table 2.2.

### 2.3.1.4 High-Resolution Optical Microscopy Methods

During the last 5 years, super-resolution optical microscopy techniques have emerged marked by the invention of the stimulated emission depletion microscopy (STED) and the stochastic optical reconstruction (STORM/fluorescence) photo-activated localization microscopy (PALM). Both tools have in common the possibility to add to the diffraction limit resolution, at least in theory, a dominator, eliminating the impossibility of resolving images smaller in size than the half of the wavelength of light. This booming resulted in the rapid development of many other high resolution improved microscopy methods, as well as the arise of many new acronyms.

High or super-resolution microscopy techniques have been classified into two groups: one based on spatially patterning the excitation light and the other based on single-molecule localization (Huang 2010). From the first group, STED microscopy is the most successful one. This technique is based on the sharpening of the laser focus with a second laser (STED laser), which suppresses spontaneous fluorescence emission by stimulated emission, as the excited fluorophore is brought to the ground state by emitting light in the same color as the STED laser. The resolution is improved when increasing STED laser intensity. Biological samples resolution has been reported to be in the range from 20 to 30 nm.

Regarding single molecule localization, this methodology is based on the detection of fluorescence of photo-switchable fluorescent probes and STORM/PALM microscopy has become one of the most known methods. This method applies several imaging cycles, where a small, optically resolvable fraction of fluorophores is activated and imaged, repeating the cycles while scanning the sample. The fundamental principle is based on the consecutive emission of sufficient photons to enable precise localization before it becomes deactivated by photo-bleaching. By this way, the resolution of the final image is not limited by diffraction, but by the precision of each localization, being possible to achieve up to approximately 20-nm resolution and with the advantage of only requiring a standard fluorescence microscope, low power continuous wave lasers, and a sensitive CCD camera (Bates et al. 2008), however their use in food materials has not been documented.

**Table 2.2.** Applications of the CLSM and multiphoton microscopy in organic and inorganic materials

Materials	Description	Reference
Bacteria	<i>Bacillus cereus</i> spores germination	Coote et al. 1995
Zein films	Zein films deformation mechanisms. Real time and microtensile stage	Emmambux and Stading 2007
Biopolymer gels	Nature and spatial distribution of protein aggregates and polysaccharides in gel structure	van den Berg et al. 2009
Emulsions	Oil-in-water (O/W) emulsion structure and stability	Calero et al. 2013
Polysaccharides	Localization of polysaccharides in foods, (pectin and carrageenan) by immunological in-situ techniques	Arloft et al. 2007
Food composite	Separation of phases in a gel during compression	van den Berg et al. 2008
Starch noodle dough	Evaluation of swelling behavior of the broccoli and starch particles in noodles	Silva et al 2013
Starch	Gelatinization behavior of starch granules obtained from different sources (maize, potato, wheat, and barley)	Schirmer et al. 2013
Dough	Wheat dough proteins microstructure and their relation with rheology properties	Jekle and Becker 2011
Foods	Potential and limitations of CLSM to study the microstructure of Yam ( <i>Dioscorea cayenensis rotundata</i> ) parenchyma, bread, and pasta (cereal products)	Dürrenberger et al. 2001
Lacteous	Effect of whey proteins on structural properties of stirred yoghurt systems at different protein and fat content	Krzeminski et al. 2011
Biofilms	Identification of bacteria and extracellular polymeric substances distribution within a biofilm matrix during growth	Wagner et al. 2009
Materials	Proliferation, morphology, and adhesion of fibroblast and epithelial cell lines, on Fe–Pd thin films	Allenstein et al. 2012 (In Press)
Seeds	Three-dimensional (3D) distribution of proteins and lipids in a parenchyma cell from seed, showing green fluorescence from protein bodies and yellow from pectin (middle lamella)	Mosele et al. 2011

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