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Original article

Multiple fluorescence labelling of proteins, lipids and whey in dairy products using confocal microscopy

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Abstract — Texture optimisation of dairy products is a major aim for manufacturers. A better knowledge of the structure and spatial organisation of their main components would allow the optimisation of their texture. In this study, using confocal scanning laser microscopy, a multiple fluorescent labelling of proteins, lipids and whey was developed to visualise these main components simultaneously in dairy products. Different extrinsic fluorescent probes were tested by confocal microscopy and fluorescence spectroscopy. Fuchsin acid, Bodipy® 665/676 and DM-NERF were selected to label proteins, lipids and whey, respectively. Methods for selecting stable and specific fluorescent probes and for obtaining the multiple fluorescent labelling are presented. An application example on a dairy gel is also shown. © Inra/Elsevier, Paris.

microscopy / fluorescence / protein / lipid / curd

Résumé — Colocalisation des protéines, des lipides et du lactosérum dans les produits laitiers par microscopie confocale. L'optimisation de la texture des produits laitiers est un enjeu majeur pour les industries. Une meilleure connaissance de la structure et de l'organisation spatiale des principaux constituants de ces produits permettrait une optimisation de leur texture. Dans cette étude, réalisée à l'aide d'un microscope confocal à balayage laser, un marquage fluorescent spécifique des protéines, des lipides et du lactosérum a été développé pour localiser simultanément ces principaux composés dans des produits laitiers. Différentes sondes extrinsèques ont été testées par microscopie confocale et spectroscopie de fluorescence. L'acide fuchsin, le Bodipy® 665/676 et le DM-NERF ont été sélectionnés pour marquer respectivement les protéines, les lipides et le lactosérum. La démarche utilisée pour sélectionner les différentes sondes stables et spécifiques et pour réaliser la colocalisation sont détaillées. Un exemple d'application sur un gel laitier est également présenté. © Inra/Elsevier, Paris.

microscopie / fluorescence / protéine / lipide / caillé

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1. INTRODUCTION

Texture optimisation of dairy products is a major aim for dairy manufacturers. This optimisation implies a better knowledge of the structure of these products. Light microscopy is a well-developed technique for studying the microstructure of food systems [8, 21]. However, this technique cannot be very well applied to the study of thick samples. Indeed, the large field depth of the light microscope produces images containing superimposed information from different focal planes and diffracted light from areas which are out of focus. Thus, it prevents the recording of images with high resolution in thick specimens [15, 16, 20]. On the other hand, confocal microscopy, mainly used in the biomedical fields, presents an important potential for the microstructure characterization of food products [1, 3, 11]. Compared to conventional fluorescence microscopy, confocal microscopy allows the recording of optical sections preventing the out-of-focus radiation. It provides a better signal-to-noise ratio and consequently an improved resolution although the confocal microscope is also limited by both laser excitation wavelengths, and by transparency of the sample [5, 15, 16, 20]. In addition to the possibility of 3-D reconstruction, multiple images of the same field can be obtained showing the spatial distribution of different chemical components, which can then be superimposed in order to visualise their relative distributions.

Although the multiple labelling for the study of the microstructure and the spatial organisation of different components in a product had some interest, this method is however seldom applied in the food field. In many studies on the structure of dairy products using fluorescence microscopy, only one of the main components, either proteins or lipids, is labelled [3, 11, 19]. It appears interesting to localise simultaneously proteins, lipids and whey. Indeed, when only proteins are labelled, it is not possible to understand if the pores are filled by lipids, whey or air. A double labelling of proteins and lipids using fluorescein isothiocyanate (FITC) and Nile Red, respectively, was reported in the literature [1]. However, fluorescence emissions of Nile Red and FITC cannot be completely separated and, consequently, an artefactual labelling of proteins is observed in the lipid fluorescence image. Moreover, FITC rapidly undergoes a photobleaching, consisting of an irreversible destruction of the excited fluorophore under high-intensity illumination conditions [10]. This phenomenon prevents the study of protein network structure for long illumination exposures, such as kinetic studies. As a consequence, the realisation of a multiple labelling and the recording of good images require that each of the fluorescent probes used presents a strict specificity for one component, does not undergo photobleaching and presents emission fluorescence spectra with a minimum of overlapping in order to recover only the fluorescence emission of each probe.

Thus, the main purpose of this study was to develop a multiple fluorescent labelling of proteins, lipids and whey in order to visualise simultaneously the different components of dairy products and to follow their evolution during milk coagulation kinetics. In the present paper, the methodology for obtaining the multiple labelling of proteins, lipids and whey on a model dairy gel is presented.

2. MATERIALS AND METHODS

2.1. Coagulation system

Raw bovine milk was purchased from a local dairy plant. Before the coagulation kinetics, the milk pH was adjusted to 6.7 using HCl 1N. Milk coagulation was performed by progressive acidification with glucono-δ-lactone (Roquette, Lestrem, France) added at the concentration of 1.75 $g \cdot L^{-1}$ The coagulation kinetics were realised at 30 °C.

Coagulations were performed inside cavity slides (800 µm). The slides were submitted to slow rotary movement during the coagulation kinetics to avoid the creaming of the fat globules. For all the experiments, the extrinsic fluorescent probes were added in the milk prior to the coagulation.

2.2. Fluorochromes and stock solutions preparation

BODIPY® 665/676 (=(E,E)-3,5-bis-(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a-diaza-s-indacene), DM-NERF and 9, 10-diphenylanthracene were from Molecular probes, Inc (Eugene, OR, USA). 1-anilino-8-naphthalene sulfonic acid (ANS), and diphenylhexatriene (DPH) were provided by Sigma (Saint-Quentin-Fallavier, France). Fuchsin acid was from Merck (Chelles, France).

The solubilization conditions and the concentrations of the stock solutions are reported in *table I*.

2.3. Confocal laser scanning microscopy

The multiple fluorescent labelling method was developed using a Zeiss LSM 410 Confocal microscope (Zeiss, Le Pecq, France) used in epi mode and fitted with the following four lasers: UV (363.8 nm), Blue (488 nm), Green (543 nm) and Red (633 nm) and mounted with 9 different filters, either Long Pass filter (LP) or Band Pass filter (BP) (*figure 1*). The BP filters allowed to recover fluorescence emission between 2 wavelengths and were used to ensure spectral discrimination.

2.3.1. Utilisation conditions of fluorophores for confocal microscopy

All the extrinsic fluorescent probes were separately screened by confocal microscopy to eliminate the non specific fluorochromes and those which were sensitive to photobleaching.

The final concentrations of fluorophores in milk, excitation lasers and filters were tested. In

Table I. Solubilisation conditions and concentrations of stock solutions of each tested probe and their utilisation conditions for confocal microscopy.

Tableau I. Conditions de solubilisation et concentrations des solutions mères pour chaque sonde testée et leurs conditions d'utilisation pour la microscopie confocale.

Tested Probes	Stock solutions	Final concentration in milk	Lasers	Filters
Protein probes				
ANS	10 mmol·L^{-1} in ethanol	10 µmol·L^{-1}	UV	BP 450–490 nm
Fuchsin acid	100 mmol·L^{-1} in water	0.4 mmol·L^{-1}	Green	BP 575–640 nm
Lipidic Probes				
9, 10-diphenylanthracene	5 mmol·L^{-1} in acetonitrile	5 µmol·L^{-1}	UV	BP 400–435 nm
Diphenylhexatriene	5 mmol·L^{-1} in acetonitrile	5 µmol·L^{-1}	UV	BP 450–490 nm
BODIPY® 665/676	65 µmol·L^{-1} in chloroform, methanol and ethanol (1V/1V/1V)	6.5 µmol·L^{-1}	Red	LP 665 nm
Whey probe				
DM-NERF	1 mmol·L^{-1} in phosphate buffer (5 mmol·L^{-1}, pH = 8)	5 µmol·L^{-1}	Blue	BP 515–565 nm

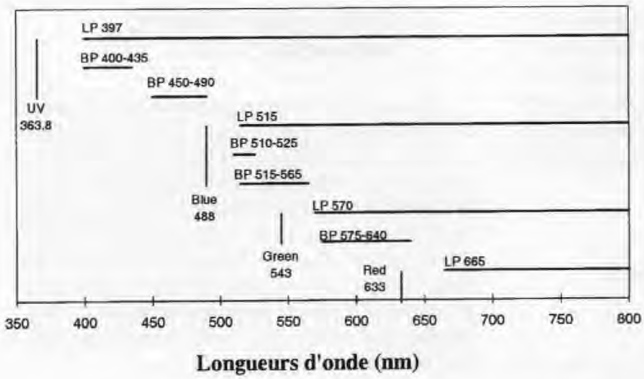

Figure 1. Lasers and filters fitted on ZEISS LSM 410 confocal microscope. Band Pass filter (BP) and Long Pass filter (LP).

Figure 1. Lasers et filtres disponibles sur le microscope confocal ZEISS LSM 410. Filtre à bande passante (BP) et filtre passe haut (LP).

table I, are presented the optimal utilisation conditions of each tested probe for confocal microscopy. It is noticed that the fluorescence intensity of Fuchsin acid is sensitive to pH, consequently the optimal concentration of this probe in milk depends on sample pH.

2.3.2. Realisation of multiple fluorescent labelling

To perform the multiple fluorescent labelling, each probe is successively excited by a laser, in the same field, and their fluorescence emission is detected with specific filters allowing to localise each of the three main components on separate images. To minimise photobleaching by high energy radiation, the sample is first excited by the red laser, then by the green laser and finally by the blue laser. To display simultaneously the three components on the same image, the fluorescence images of each component (grey levels) are transferred in different channels called Red, Green and Blue channels, attributing for each component a false colour (red, green and blue, respectively). These images are superimposed in a red/green/blue (RGB) channel yielding a single image with each labelled compound displayed by the false colour previously determined.

2.4. Recording of excitation and emission spectra by front face fluorescence spectroscopy

Fluorescence spectra were recorded using a SLM 4800 spectrofluorimeter (Bioritech, Chamarande, France) provided with a thermostated front-surface accessory. Considering the cuvette holder, the incidence angle of the excitation radiation was 60°. All spectra were corrected for instrumental distortions in excitation using a rhodamine cell in reference channel.

It is well known that the fluorescence properties of molecules depend on their environment. Consequently, for each selected probe by confocal microscopy, the excitation and emission spectra were recorded in the milk in order to select the appropriate filters to specifically detect the fluorescence emission of these probes. DM-NERF, Fuchsin acid and Bodipy® 665/676 were added to the milk at a final concentration of 2.5 $\mu mol \cdot L^{-1}$, 0.5 $mmol \cdot L^{-1}$ and 3.25 $\mu mol \cdot L^{-1}$, respectively. The excitation spectra of DM-NERF (420–530 nm), Fuchsin acid (445–555 nm) and Bodipy® 665/676 (600–685 nm) were recorded with emission wavelengths set at 534, 620 and 705 nm, respectively and the emission spectra of DM-NERF (515–650 nm), Fuchsin acid (565–650 nm) and Bodipy® 665/676 (670–720 nm) were recorded with excitation wavelengths set at 509, 510 and 633 nm, respectively.

3. RESULTS AND DISCUSSION

To perform a multiple labelling, intrinsic or extrinsic fluorescent probes must be used to discriminate the different components. In milk, the tryptophan residues and vitamin A may be considered as the most appropriate intrinsic fluorescent probes, which are specific of proteins and lipids, respectively [6]. However, theses probes cannot be excited by lasers installed on the confocal microscope because the lowest excitation ray of lasers is located at

363.8 nm, while tryptophan excitation spectrum spreads between 250 and 310 nm and vitamin A between 270 and 350 nm. Consequently, extrinsic fluorescent probes must be used to observe proteins, lipids and whey in milk.

3.1. Selection of fluorescent extrinsic probes using confocal microscopy

As described previously, to perform a multiple fluorescent labelling, fluorophores must specifically label the different components and, as far as possible, without photobleaching. However, most fluorophores are used in a simple medium (for spectroscopic studies) and under illumination conditions of low intensity. Thus, a first selection of probes was made using confocal microscopy to eliminate the non specific probes and those which were sensitive to photobleaching.

Six fluorophores known to label either proteins or lipids or being used as pH indicators and commonly used in spectroscopy and microscopy studies were investigated (*table II*). To label protein network, ANS was first tested. This probe is frequently used in fluorescence spectroscopy studies to label proteins [12, 13, 17, 18]. However, ANS photobleached upon the UV light exposition. Moreover, this probe also labelled lipid compounds. Indeed, on the fluorescence image obtained with ANS, the diffuse structures were labelled as well as the round structures. In the literature, lipids are described as round structures (globules), while proteins are reported as diffuse structures. Consequently, this probe is not specific to proteins and it is impossible to discriminate the protein network and the fat globules. Another probe known to label proteins [7], Fuchsin acid, was then tested. Fuchsin acid was revealed to be specific to proteins and underwent no photobleaching. Fuchsin acid was selected for the labelling of proteins. Although FITC is a dye frequently used to label proteins in microscopy [1, 3], this probe was not been screened in this study. Indeed, this fluorophore is well known to photobleach, to be excited by the blue and green lasers and to present a large fluorescence emission spectrum yielding difficult the specific detection of fluorescence emission of another probe [1, 10].

Table II. Fluorescent extrinsic tested probes for labelling proteins, lipids and whey.

Tableau II. Sondes fluorescentes extrinsèques testées pour le marquage des protéines, des lipides et du lactosérum.

Tested Probes	Lasers	Observations
Protein probes		
ANS	UV	Not specific of proteins and photobleaching
Fuchsin acid	Blue and green	Specific of proteins and specific detection of fluorescence emission ➨ Selected
Lipidic probes		
9,10-diphenylanthracene	UV	Specific of lipids but photobleaching
Diphenylhexatriene	UV	Specific of lipids but photobleaching
BODIPY® 665/676	Red	Specific of lipids and specific detection of fluorescence emission ➨ Selected
Whey probe		
DM-NERF	Blue	Specific of whey and specific detection of fluorescence emission ➨ Selected

To label the lipids, very apolar probes, as 9,10-diphenylanthracene [10] and DPH [4, 14, 18] were tested. These probes, specific for lipids, presented severe photobleaching upon UV light excitation. In fact, it appeared that most of the fluorescent probes photobleached upon exposure to high energy of UV laser. Consequently, fluorophores excited by the UV laser were discarded from the screening process. Bodipy® 665/676 that offers an unusual combination of apolar structure and long-wavelength fluorescence was then tested [10]. This probe was excited by the red laser, revealed to be a specific dye for lipids and stable upon illumination. Considering these characteristics, Bodipy® 665/676 was selected for labelling of lipids. In the literature, it appears that Nile Red [1, 3] and Nile Blue [11, 21] are the fluorophores the most frequently used in microscopy to stain lipids. But, these probes, as well as FITC, can be excited by the blue and green lasers and present a large emission spectrum. These fluorescent properties did not match well with the multiple fluorescence labelling approach developed in this study.

To reveal whey, DM-NERF was tested. This probe is usually used for monitoring pH by fluorescence spectroscopy [10]. It was excited with the blue laser, labelled only the aqueous phase and presented no photobleaching. DM-NERF was selected to localise the whey.

The three selected probes to specifically label proteins, lipids and whey are Fuchsin acid, Bodipy® 665/676 and DM-NERF, respectively.

3.2. Selection of filters for the specific fluorescence emission detection of each probe using front face fluorescence spectroscopy

In order to confirm the selection of fluorophores by confocal microscopy, the fluorescence emission of these probes must be specifically detected by appropriate filters to simultaneously display proteins, lipids and whey. It is well known that the excitation and emission wavelengths of the fluorophores as well as their quantum yield, depend on their environment [9]. For these reasons, it appeared essential to record the fluorescence spectra of the selected probes in raw milk, which was achieved by front face fluorescence spectroscopy. These data made it possible to choose the appropriate lasers and filters for confocal microscopy.

The excitation and emission spectra of DM-NERF, Fuchsin acid and Bodipy® 665/676 are presented in *figure 2*. For DM-NERF, the excitation and emission spectra spread from 420 to 530 nm and from 520 to 650 nm, respectively. In the case of Fuchsin acid, excitation and emission spectra spread from 445 to 555 nm and from 565 to 650 nm, respectively. Fuchsin acid displays a maximum excitation around 540 nm, in agreement with literature [7], but this probe may also be excited by the blue laser at 488 nm (*figure 2*). Thus, the blue laser excites both DM-NERF and Fuchsin acid. It is therefore necessary to specifically recover the fluorescence emission of the DM-NERF by a Band Pass filter between 515 and 565 nm. Since DM-NERF and Fuchsin acid display distinct emission spectra, it was possible to specifically detect their fluorescence emission. Fuchsin acid was excited at 543 nm by the green laser and fluorescence emission was detected between 575 and 640 nm with a Band Pass filter. In the case of Bodipy® 665/676, the excitation spectrum spreads from 600 to 685 nm and the emission spectrum from 670 to 720 nm. Bodipy® 665/676 was excited at 633 nm with the red laser and fluorescence emission was detected above 665 nm with a Long Pass filter. Since the fluorescence emission of the three probes selected for confocal microscopy can be specifically recovered, DM-NERF, Fuchsin acid and Bodipy® 665/676 were used to visualise whey, proteins and lipids, respectively, in a dairy gel.

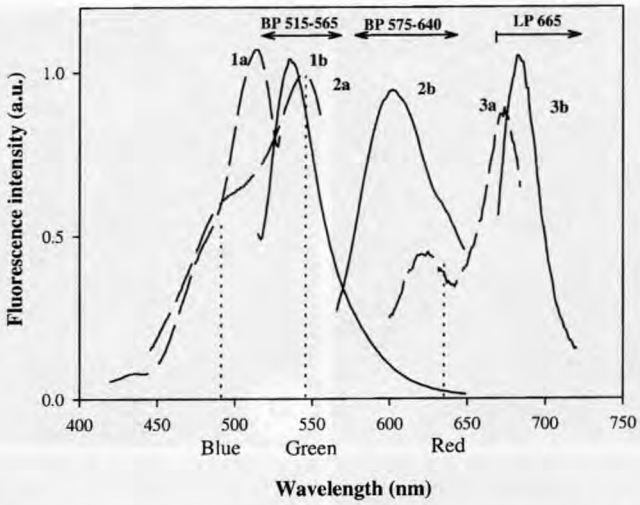

Figure 2. Excitation and emission spectra for DM-NERF, Fuchsin acid and Bodipy® 665/676 probes and their utilisation conditions for confocal microscopy (lasers and filters). Spectra obtained in the milk using front face fluorescence. (– – –): excitation spectra of DM-NERF (**1a**), Fuchsin acid (**2a**) and Bodipy® 665/676 (**3a**); (—) : emission spectra of DM-NERF (**1b**), Fuchsin acid (**2b**) and Bodipy® 665/676 (**3b**).

Figure 2. Spectres d'excitation et d'émission du DM-NERF, acide fuchsine et Bodipy® 665/676 et leur condition d'utilisation pour la microscopie confocale (lasers et filtres). Spectres obtenus dans le lait utilisant la spectroscopie de fluorescence frontale. (– – –) : spectres d'excitation du DM-NERF (**1a**), acide Fuchsine (**2a**) et Bodipy® 665/676 (**3a**) ; (—) : spectres d'émission du DM-NERF (**1b**), acide fuchsine (**2b**) et Bodipy® 665/676 (**3b**).

3.3. Multiple fluorescent labelling of proteins, lipids and whey in a dairy gel

In order to validate the developed method, a multiple labelling of proteins, lipids and whey was performed on a dairy gel. Images of the fat globules stained with Bodipy® 665/676, protein network labelled with Fuchsin acid and whey stained with DM-NERF were visualised, respectively, in the red channel (*figure 3a*), in the green channel (*figure 3b*) and in the blue channel (*figure 3c*). In *figure 3d* is presented the triple-labelling image allowing to localise simultaneously the main components of the gel in the RGB channel. On this image, the fat globules appeared in red, the protein network appeared in cyan (not in green as *figure 3b*) and the whey in dark blue. The labelling of the protein network in cyan results from casein micelle hydration [2] involving the superposition of green and blue colours.

4. CONCLUSIONS

The multiple labelling of proteins, lipids and whey allows the study of the microstructure and the simultaneous localisation of these components. Moreover, since the images of each component are recorded separately, a quantification of their texture may be performed by different texture image analysis techniques. This method will be used in further study to monitor the porosity of the protein network during different milk coagulation processes.

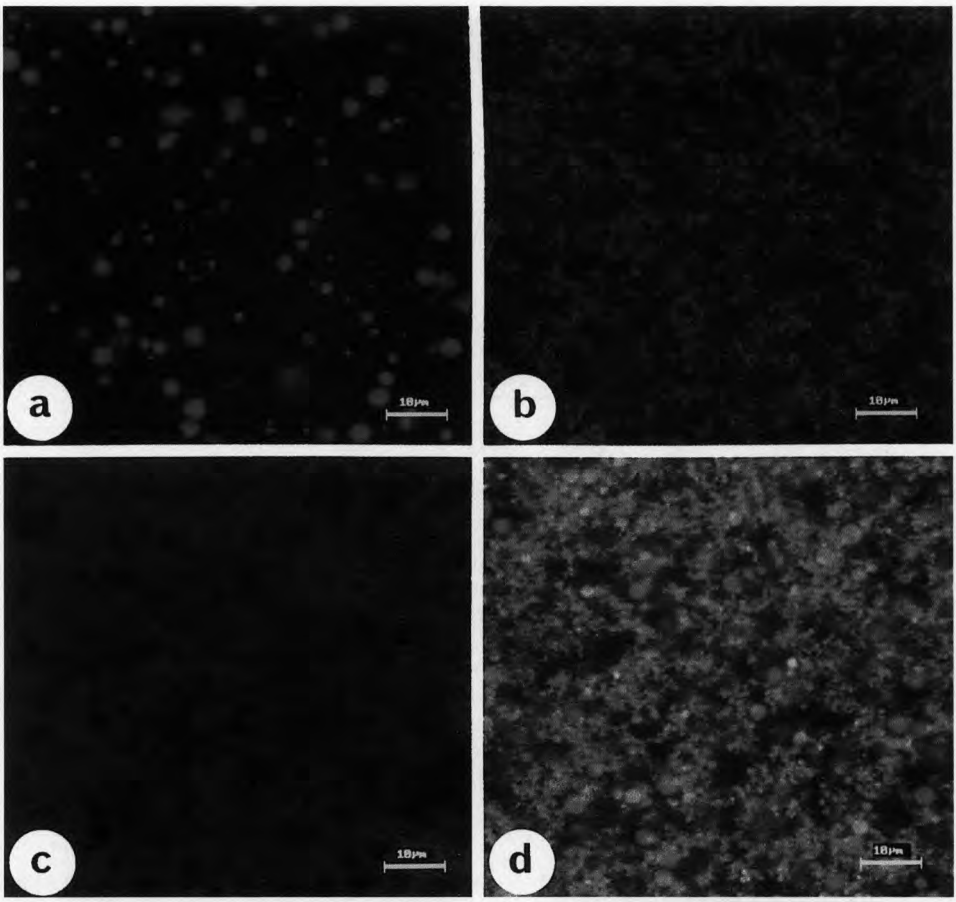

Figure 3. Fluorescence image of fat globules (Red channel) (**a**), protein network (Green channel) (**b**), and whey (Blue channel) (**c**). The three images were superimposed in the red/green/blue channel (RGB) giving the triple-labelling image (**d**).

Figure 3. Image de fluorescence des globules gras (canal rouge) (**a**), du réseau protéique (canal vert) (**b**), et du lactosérum (canal bleu) (**c**). Superposition de ces trois images dans le canal rouge/vert/bleu (RVB) donnant l'image du triple marquage (**d**).

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