Protocol for Laser Scanning Microscopy of Microorganisms on Hydrocarbons

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Abstract

Microbial communities in their fully hydrated state can be imaged in space and time (4-dimensionally) by laser scanning microscopy using 1-photon or 2-photon excitation. In this protocol, we provide guidance on how to examine microorganisms associated with liquid, viscous and solid hydrocarbons. Practical aspects are discussed including the material and sources, microscopy consumables, software programs and time constraints. The details of mounting samples for the upright and inverted microscope as well as options for fluorescence staining of bacteria and hydrocarbons are presented. Suggestions are made for recording images and subsequent digital image analysis. Finally, notes are added and a guideline for troubleshooting is supplied.

Keywords: Bacteria, Biofilms, Colonisation of hydrocarbons, Confocal laser scanning microscopy, Deconvolution, Degradation of hydrocarbons, Digital image analysis, Fluorescence techniques, Fluorochromes, Hydrophobicity, Image analysis, Imaging, Imaging techniques, Laser scanning microscopy, Lectins, Microorganisms, Quantification, Two photon laser scanning microscopy, Visualisation

1 Introduction

Laser scanning microscopy (LSM) represents an established technique for structure–function studies of microbial aggregates and films. The main advantage of LSM is its 3-dimensional sectioning capability of fully hydrated, living microbial communities. The LSM approach allows multichannel imaging of cellular constituents and extracellular polymeric substances (EPS). In addition, the microenvironment can be examined using a variety of fluorescent probes. With state of the art instruments, up to six different parameters can be recorded simultaneously or sequentially. The digital image series recorded may then be used for visualisation and quantification. The basics of LSM and details of how to apply LSM in order to examine a microbiological sample in general are out of the scope of this protocol. However, there is an extensive series of reviews describing the applicability of LSM with focus on microbial

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communities such as biofilms and bioaggregates (*see* **Note 1**). The focus of this review is on LSM and its suitability to examine the interaction of microorganisms with hydrocarbon interfaces.

The interaction of microorganisms with hydrocarbons may occur directly or via surface active compounds. In fact, microbial surface active compounds maybe involved not only in the degradation of hydrocarbons but also in the interaction of microorganisms (adhesion and detachment) with interfaces [1]. In many cases, the microbial communities degrading hydrocarbons are located in close proximity to the hydrophobic substrate which at the same time may serve as a substratum. Consequently, the microorganisms adhere to solid hydrocarbons as well as to liquid hydrocarbons and develop into a microbial biofilm. Thereby microorganisms, especially if they possess a hydrophobic cell surface, may stabilise oil–-water emulsions [2]. The necessity to image the adhesion of bacteria to hydrocarbons at different depths of focus is apparent from an example recorded by conventional light microscopy (*see* Fig. 1 in Rosenberg and Doyle [3]). They showed bacteria colonising a spherical hydrocarbon droplet and demonstrated the ability to focus in one optical layer only, thereby indicating the need for 3D imaging of fully hydrated samples. Nevertheless, microbial colonisation of hydrocarbons has been investigated by LSM in a few studies only.

For example, Whyte et al. studied the degradation of hexadecane and diesel fuel by a *Rhodococcus* strain at low temperature [4]. They could show the bacteria colonising the water–hydrocarbon interface of the microdroplets. By fluorescence lectin-binding analysis (FLBA) the different glycoconjugates produced were related to the temperature and carbon source employed. They finally demonstrate that the strain can assimilate both solid and liquid hydrocarbons. Some of the data was impressively visualised 3-dimensionally in a later review article [5]. The group of Baldi investigated the interaction of *Acinetobacter venetianus* with diesel fuel droplets [6]. It was shown that adhesion to the hydrocarbon droplets involved a glycoconjugate which was stained by lectins. Using LSM time series, they were able to demonstrate two types of interactions: (1) cell–cell interactions before colonising the hydrocarbon and (2) incorporation of nanodroplets into the capsular polysaccharide. Another manuscript by the same group reported the growth of a yeast strain, *Rhodosporidium toruloides*, on dibenzothiophene and orimulsion [7]. By means of LSM, the growth of yeast on hydrocarbons, the changes in morphology and the production of glycoconjugates were followed. Macedo et al. employed LSM to follow the colonisation of polychlorinated biphenyls (PCB) droplets by a microbial community isolated from PCB-contaminated soil [8]. For this purpose, they used a variation of the hanging drop cultivation method. For examination by LSM, the PCB droplets on plastic slides were

studied using an upright LSM system with water-immersible lenses. Thereby pronounced stages of microbial colonisation could be established which correlated with the degradation of the complex PCB mixture. In a follow-up manuscript, the adaptation of the community to different PCB levels was studied [9]. LSM assessment of the PCB samples showed different types of biofilms, starting from thin layers on the PCB droplets towards more aggregated layers containing dense microcolonies. In both studies, the extracellular hydrocarbon phase was stained with Nile Red, whereas in a study on bacterial hydrocarbon production, Nile Red was used to stain the cellular hydrocarbon fraction [10]. The colonisation of polycyclic aromatic hydrocarbons (PAH) in flow cells was investigated with triple-species biofilms. For LSM imaging of bacterial species, a combination of GFP (*Sphingomonas*), DsRed (*Pseudomonas*) and Syto 62 (*Mycobacterium*) was employed. PAH was imaged via its autofluorescence under UV excitation [11]. Similarly, the tolerance of bacteria against solvents can be studied using LSM. For this purpose, *Pseudomonas* biofilms were exposed to styrene and examined with respect to biofilm structure, membrane damage and glycoconjugate production [12]. In a field study, the in situ bioremediation of a hydrocarbon-polluted site was stimulated by adding hydrogen peroxide, an oleophilic fertiliser and a surfactant. The experiment was followed by chemical analysis of hydrocarbons, traditional enrichment techniques for bacteria and LSM [13].

Most of the studies used a similar LSM approach to examine the interaction of the microorganisms with hydrocarbons. For fluorescence staining, Nile Red was used for the liquid hydrocarbon phase, a nucleic acid stain (e.g. Syto 9) for the bacteria and various lectins for visualisation of the glycoconjugates. The details of the staining procedures are described below.

2 Materials

2.1 Internet Sites and Sources

2.1.1 Microscopy Supplies

- http://www.marienfeld-superior.com/index.php/cover-glasses/articles/precision-cover-glasses-thickness-no-15h-for-high-performance-microscopes.html
- https://de.vwr.com/app/catalog/Product?article_number=737-0013
- http://www.gracebio.com
- http://www.thermoscientific.com/en/product/nunc-lab-tek-chambered-coverglass.html
- http://www.emsdiasum.com/microscopy/products/preparation/imaging-microscopy.aspx
- http://www.finescience.de/index.asp?verified=true

2.1.2 Fluorochromes and Lectins

Fluorochromes

- http://www.lifetechnologies.com/de/de/home/brands/molecular-probes.html
- http://www.biostatus.com
- http://www.dyomics.com/
- http://www.atto-tec.com/
- http://www.biotium.com
- http://www.abberior.com

Lectins

- http://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html?TablePage=17904091
- http://www.eylabs.com/index.php?page=shop.browse&category_id=6&option=com_virtuemart&Itemid=79
- https://www.vectorlabs.com/catalog.aspx?catID=31&locID=0

2.1.3 Commercial Visualisation and Deconvolution Programs

Visualisation

- Imaris – http://www.bitplane.com
- Amira – http://www.amiravis.com
- Volocity – http://www.improvision.com
- Image-Pro – http://www.mediacy.com/index.aspx?page=Image_Pro_Software
- MetaMorph – http://www.moleculardevices.com/Products/Software/Meta-Imaging-Series/MetaMorph.html

Deconvolution

- Huygens – http://www.svi.nl/HomePage
- AutoQuant – http://www.mediacy.com/index.aspx?page=autoquant
- Volocity – deconvolution as option
- CLSM software – most companies offer a deconvolution option

2.1.4 Freely Available Programs

General

- ImageJ – http://rsb.info.nih.gov/ij/
- Fiji – http://fiji.sc/fiji

Developed for Microbiological Data Sets

- Comstat 1 and 2 – http://www.comstat.dk/
- Phlip – http://sourceforge.net/projects/phlip/
- Daime – http://www.microbial-ecology.net/daime/

Visualisation

- BioImageXD – http://www.bioimagexd.net/
- ImageSurfer – http://imagesurfer.cs.unc.edu/
- VolViewer – http://cmpdartsvr3.cmp.uea.ac.uk/wiki/BanghamLab/index.php/VolViewer

Deconvolution

- Plug-in for ImageJ – http://bigwww.epfl.ch/algorithms/deconvolutionlab
- BiaQIm – http://www.deconvolve.net/index.html

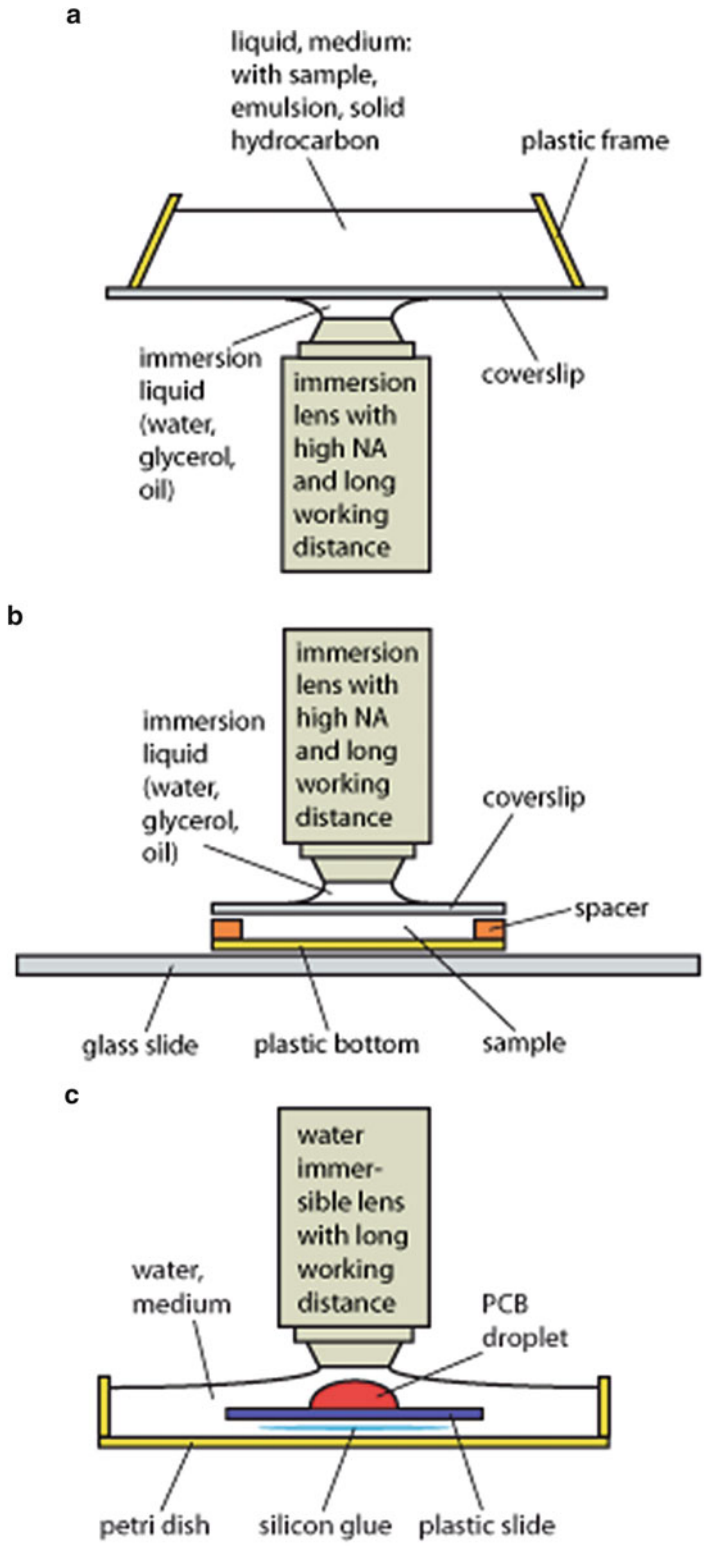
3 Methods

The procedures will describe how to examine microbial communities growing on hydrocarbons by using LSM. A differentiation is made in terms of studying bacteria on liquid, viscous and solid hydrocarbons. The approach is split into sample mounting, staining, collecting images and data analysis.

3.1 Growing Microorganisms on Hydrocarbons with Respect to LSM

There are different approaches regarding how to grow microbial communities on hydrocarbons. The main issue in terms of growth and LSM examination is the consistency of the hydrocarbon, meaning whether it is liquid, viscous or solid at the temperature employed for cultivating the microorganisms.

1. If the hydrocarbon is liquid, the culture can be performed in a normal Erlenmeyer flask containing a simple mineral medium.
2. If the hydrocarbon is viscous, a variation of the hanging drop method maybe used. For this purpose, a droplet of the hydrocarbon is put onto a hydrophobic plastic slide which is exposed to the microbial community.
3. If the hydrocarbon is used in the form of waxy material or solid crystals, they also may be added to an Erlenmeyer flask with mineral medium. Another option may be flow-through cells, e.g. in those applications where dissolved hydrocarbons are studied [14]. For hydrocarbons which are solid at room temperature, the substratum may be coated by the dissolved hydrocarbon with subsequent evaporation of the solvent [11]. Another option would be that solid crystals are mounted in a flow-through cell in order to follow biofilm development over time, although this, to the best of our knowledge, has not been published.


a
liquid, medium: with sample, emulsion, solid hydrocarbon
plastic frame
immersion liquid (water, glycerol, oil)
immersion lens with high NA and long working distance
coverslip
b
immersion lens with high NA and long working distance
immersion liquid (water, glycerol, oil)
coverslip
spacer
glass slide
plastic bottom
sample
c
water immer-sible lens with long working distance
water, medium
PCB droplet
petri dish
silicon glue
plastic slide

3.2 Mounting of the Sample for LSM

1. First, a subsample of the culture matching the LSM requirements has to be collected. In liquid cultures, collect the emulsified hydrocarbon with the associated bacteria using an inverted glass pipette (5 or 10 mL) in order to apply as little shear force as possible. Place the emulsion in the appropriate chambers, commercial or self-made (*see* **Note 1**). The same procedure can be used if the bacteria grow on solid hydrocarbons. In that case, a few hydrocarbon crystals with the attached bacteria are transferred into a suitable chamber.
2. The mounting of the sample will be determined by the LSM type available, upright or inverted microscope (Fig. 1). For both microscope types, apply the staining in the chamber used. For the *inverted* setup, place the sample in a coverslip chamber and image it from below (Fig. 1a). The chambers are available with 1, 2, 4 or 8 wells having different sizes. In this case, the free working distance of the objective lens maybe a problem if hydrocarbon droplets are rather large or due to the fact that hydrocarbons will float on the aqueous phase or sorb to the chamber frame made from plastic. For the *upright* setup, place the sample in a cover well chamber, cover it with a coverslip and put it on a slide (Fig. 1b). It is a good idea to put a droplet of water on the slide to prevent the chambers from gliding off the slide. These chambers are available with different spacers of 0.2, 0.5, 1 and 2 mm thickness. Be aware that the hydrocarbon droplets may sorb to the coverslip. The upright setup has more flexibility and also allows mounting of larger samples, e.g. hydrocarbon-contaminated objects, in a 5 cm Petri dish. In this case, the observation can be made using water-immersible objective lenses. They have the advantage of an extra-long working distance (*see* **Note 2**).
3. For bacteria growing on viscous hydrocarbons, the plastic slide with the hydrocarbon droplet is transferred into a 5 cm Petri dish. The slide is placed in the dish with the droplet facing upwards. In order to avoid floating of the plastic slide, it can be glued to the Petri dish using silicon adhesive (RTV silicone, WPI, Sarasota, FL). This type of mounting is suitable for imaging with the upright microscope only (Fig. 1c). For an inverted microscope, it is more difficult as the plastic slide has

Fig. 1 Mounting options for examination of hydrocarbon-associated microorganisms by means of LSM. (**a**) Setup for the inverted microscope by using coverslip chambers. The rectangular chambers are available with plastic frames creating wells of different sizes. (**b**) Setup for the upright microscope by using cover well chambers. The square chambers are available with spacers of different height. (**c**) Special setup for the upright microscope which was used to image biofilms developing on PCB droplets [8]. A similar setup maybe used to image bacteria or fungi growing on solid hydrocarbons

to be mounted upside down in a coverslip chamber using self-made spacers. The spacers may be of different materials (*see* below) and have to be used in order to avoid squeezing of the droplet with the attached bacteria.

3.3 Staining Solutions: Nucleic Acids (Bacteria)

1. Nucleic acid-specific fluorochromes (e.g. Syto 9, Life Technologies) are usually delivered in vials containing 100 µL at a concentration of 5 mM in DMSO. Prepare aliquots of 2 µL and store at −20°C for later use (*see* **Notes 3** and **5**).
2. For staining, take an aliquot and dilute it in 2 mL of appropriate buffer, medium or, e.g. filtered river water. Staining of bacteria is done by adding a few droplets to the sample and incubating for 5 min; the sample can then be examined visually or by laser microscopy without any destaining step.
3. The staining solution may be used up for a few days. Be aware that all fluorochromes must be handled with care. Most of them have not been investigated in terms of their toxicity or carcinogenicity.

3.4 Stains: Liquid/Viscous Hydrocarbons

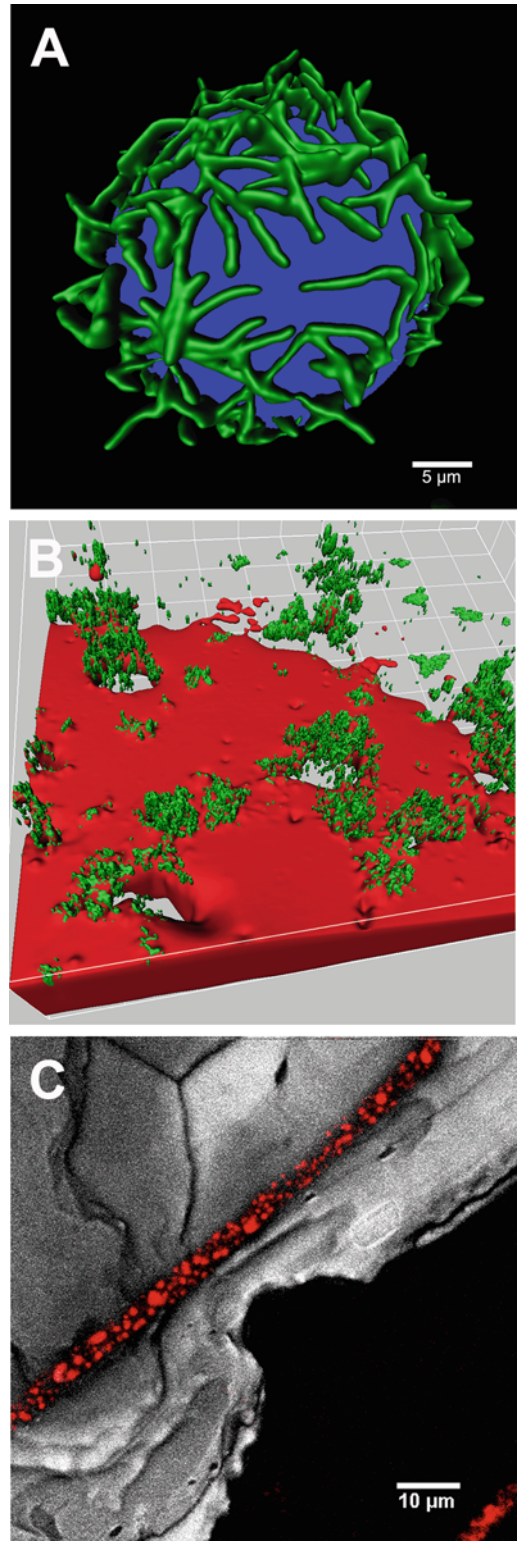
1. As a general stain for hydrocarbon compounds, the lipophilic fluorochrome Nile Red (Sigma-Aldrich) can be used. Prepare stock solution of 1 mg Nile Red in 1 mL acetone. For staining, dilute at 1:1,000 with water and incubate for 15 min (*see* **Note 3**).
2. Be aware that Nile Red will also stain lipophilic compartments, e.g. in eukaryotic microorganisms and bacteria (*see* **Note 4**).

3.5 Probes for Glycoconjugates: Lectins

1. Lectins dissolved in buffer are either supplied as solution (1 mg/mL) or freeze-dried in 1 mg portions. Freeze-dried lectins are prepared at 1 mg/mL by just adding water. From the stock solution, prepare aliquots of 100 µL and freeze at −20°C. Take 100 µL and dilute 1:10 with filter-sterilised water, buffer or medium. For staining, add a few droplets to the sample and incubate for 20 min. Carefully wash the sample 3–4 times in order to remove unbound lectins.
2. Warning – some lectins are extremely toxic and have to be handled with caution. Ideally, lectins are only handled in solution.

3.6 Fluorescence Staining Strategies

The staining approach depends on the sample properties and the aims of the study. In any case, usually the bacteria will be stained using a nucleic acid-specific fluorochrome. For this purpose, there is a choice of many fluorochromes with different excitation and emission characteristics. A particularly suitable one is Syto 9 which has been successfully employed for many different sample types. Similarly SYBR Green may be applied. In addition, it is useful to differentially image the hydrocarbon phase. If the hydrocarbon is liquid or viscous, it can be stained with a lipophilic fluorochrome such as Nile Red (Fig. 2a, b). This combination would result in a


A
5 µm
B
C
10 µm

dual-channel image with the hydrocarbon stained red and the bacteria stained green. In the case of solid hydrocarbons, the surface of the crystals can be imaged in the CLSM reflection mode (Fig. 2c). If a UV laser or a two-photon laser is available, the autofluorescence of many hydrocarbons can be imaged and recorded in a separate channel. This makes sure that the nucleic acid-stained bacteria are not lost in space but can be related to a matrix or surface. A critical issue is the potential application of fluorescence in situ hybridisation (FISH) as the chemicals used for fixation and dehydration may interfere with the hydrocarbon phase.

1. First, stain the hydrocarbon droplets with Nile Red (about 15 min), and then apply the nucleic acid-specific counterstain for the bacteria. After staining with Syto 9, you can use the sample directly for imaging. If fluorescence lectin-binding analysis (FLBA) is employed, the Syto 9 counterstaining is done last.
2. Depending on the sample properties, additional stains can be applied. For example, the glycoconjugates of the extracellular polymeric substances (EPS) maybe stained using lectins [15]. The detailed procedure of fluorescence lectin-binding analysis (FLBA) has been described elsewhere [5]. In this case, the lectin staining is done first, and then the nucleic acid-specific counterstain is added.

3.7 Collecting Images by Laser Scanning Microscopy

Laser scanning microscopy can be done in many different ways. There is not one correct way of doing LSM, but it rather depends on the purpose of imaging. Ideally the user should consult a laser microscopy specialist in order to discuss the options for collecting

Fig. 2 Laser scanning microscopy of bacteria and fungi associated with hydrocarbons of different consistency (liquid, viscous and solid). (**a**) Bacteria adhering to a suspended liquid hydrocarbon droplet. The bacteria at the hydrocarbon–water interface were stained with the nucleic acid-specific fluorochrome Syto 9. The image series of the nucleic acid signal is shown as an isosurface projection. The diesel droplet in the centre is indicated by an artificial projection of a sphere. Related images showing different projections have been published [4]. (**b**) PCB-degrading microbial community isolated from soil. The viscous PCB droplet absorbed to a plastic slide was stained with Nile Red (*red*); the bacteria were stained with SYBR Green (*green*). The data set is presented as an isosurface projection. Take notice of the microcolonies above the PCB lesions indicating the degradation of PCB by the microbial community. Grid size = 50 μm. Further details on the pronounced stages of PCB colonisation may be found in Macedo et al. [8]. (**c**) Fungal filament of *Pythium ultimum* adhering to a solid phenanthrene crystal. The fungus was stained with Nile Red indicating intracellular hydrophobic vesicles. The image was collected by means of two-photon laser scanning microscopy. Excitation was at 800 nm; the emission was recorded in two channels at 400–502 nm (autofluorescence of phenanthrene) and 587–800 nm (Nile Red)

images. A sound basis for understanding the advantages and disadvantages of laser microscopy is usually "taking the course". From experience, it is known that it may take several weeks or even months before one can use a laser scanning microscope properly and effectively. In the following, the main issues which are important for doing laser scanning microscopy are listed. For more details, *see* references in Table 1.

1. In many imaging facilities, the instrument has to be booked in advance. Usually there is a short introduction to the instrument. There might be a charge for using the microscope. Stains may have to be purchased from your own budget.
2. Look up the technical details of the laser scanning microscope. Check for upright/inverted microscope, existing lasers and laser lines for excitation, number of photomultipliers/detection

Table 1
Review articles and book chapters (only) describing laser scanning microscopy and fluorescence techniques as tools for examination of microbial communities associated with interfaces

Focus of article	References
LSM of adherent microorganisms	Gorman et al. [16]
Imaging of biofilms	Lawrence et al. [17]
Confocal laser scanning microscopy	Lawrence and Neu [15]
Fluorescence lectin-binding analysis	Neu and Lawrence [18]
In situ detection of EPS	Neu and Lawrence [19]
Confocal and other approaches	Palmer and Sternberg [20]
Structured LSM approach	Neu and Lawrence [21]
LSM with focus on immunofluorescence	Schmid et al. [22]
1-Photon versus 2-photon LSM	Neu and Lawrence [5]
Spatiotemporal approaches	Palmer et al. [23]
LSM of aggregates and flocs	Lawrence and Neu [24]
LSM applications in microbiology	Lawrence and Neu [25]
Analytical imaging	Lawrence et al. [26]
LSM of microorganisms on hydrocarbons	Neu and Lawrence [27]
LSM of biofilm matrix	Neu and Lawrence [28]
LSM of biofilm structure	Neu and Lawrence [29]

channels, objective lenses available and suitable sample chambers for microscopy (*see* **Note 6**).

3. At this stage, it might be a good idea to discuss possible software to be used for subsequent digital image analysis. Issues for directly using these programs include file loading and import of instrument parameters. Especially check the bit depth of the recorded data. Often it is 8 bit (0–255 pixel intensities) which can be handled by most image analysis programs. Many sources claim that 12 bit or 16 bit is required for scientific image data sets. However, this may cause problems in terms of the image analysis programs used at a later stage.
4. Be aware of the number of samples and the time needed for imaging (*see* comments at the end of the chapter). Usually recording one data set per sample is not enough. Often an overview is imaged and then several locations are examined at high resolution. Mostly the quality of image data is increased during recording by continuously optimising the settings. Be aware that if you work fast for a full day at the LSM, you may create 1–2 or even more GB of data.
5. Become familiar with using the laser scanning microscope. For that purpose, a variety of fluorescent beads maybe employed as test samples. Try single and multichannel imaging. Take notes of the main instrument settings (*see* **Note 7**).
6. Think about the purpose of imaging. What is the aim of collecting images? For example, 2D, 3D or 4D data sets, visualisation only, one perfect image, routine imaging of many samples, quantification, statistics and deconvolution. These aspects will determine the approach to recording images and the settings to be used.

3.8 Analysis of 3-Dimensional Image Data Sets

Digital image analysis comprises *firstly*, *visualisation* and projection of data, and *secondly*, *quantification* and extraction of numbers. For these goals, different software packages are required. Furthermore, commercial software and freely available software may have to be considered and evaluated. In addition, deconvolution may be applied in order to increase the resolution. Although it is often very appropriate to do, in most (microbiological) cases, deconvolution has not been used due to several critical issues.

1. *File formats*
   Make sure that the microscopy file format of the images is compatible with the program used for digital image analysis. Most often, the first problem encountered is the impossibility of loading the data into a particular program. Many commercial programs will have a reader for the microscopy format of the main laser microscopy companies. However, sometimes, the readers are not up to date as the microscope companies

keep changing their file formats. With most LSM software, the data can be stored in a neutral file format. Another frequent requirement is renaming of the image series. Free programs for renaming of image series can be found in the Internet.

2. Digital image analysis is a multistep procedure. One major step is thresholding of the images (*see* **Note 8**). By this procedure, a grey level image (e.g. 8 bit having 0–255 pixel intensities) is transformed into a binary image with only black and white pixels (0 and 255 pixel intensities). The thresholded image series is then used for visualisation or quantification. This key step in the procedure is considered controversially, and as a result several publications from the microbiology field should be consulted to understand the issues [30–33].

3. *Visualisation*
   Currently, most programs controlling and running the LSM instrument have several basic tools available for visualisation of the data recorded. In addition, several advanced programs specifically developed for LSM data sets are available including Amira, Imaris and Volocity, among others. They usually can be purchased as a basic program with additional add-on tools as options for specific analyses. Furthermore, there are programs freely available including, e.g. ImageJ [34] and its extension for biological imaging called Fiji [35] as well as BioImageXD [36].

4. *Quantification*
   The most popular program is again the freely available software ImageJ developed at the National Institutes of Health (NIH). At the NIH website and due to the many users, a long list of macros and plug-ins are available. Other free software developed for microbiological data include Comstat, Phlip and Daime. However, these three programs were developed for a specific microscopy file format, for certain types of samples and for special analysis procedures. As a consequence, they may be not suitable for all types of data sets. Again, often loading the data is an issue, and renaming the files may be required if these software packages are employed.

5. *Deconvolution*
   Deconvolution is applied in order to (a) remove noise from an image data set, (b) to enhance the resolution in XY and (c) to improve axial (XZ) elongation. However, for correct calculations, the images have to be recorded at the Nyquist rate, meaning at a pixel resolution of 50 nm and a step size of about 150 nm. Both will lead to dramatic bleaching if an image stack with a large number of sections has to be recorded. In addition, the point spread function of the instrument has to be measured, ideally inside the actual sample to be analysed. As

a consequence, deconvolution has been applied in only a few studies. Nevertheless, a comparison of the two major software packages has been published [37].

3.9 Time Considerations

Laser microscopy is a 3-step procedure: (1) sample mounting and staining, (2) recording of images and (3) digital image analysis (quantification and visualisation). With fresh samples, mounting and staining are straightforward and can be done quickly (time frame 5–30 min). If samples have to be fixed, embedded and sectioned, it may take 1–2 days. The use of cryo-sectioning techniques may reduce this time to half a day. A similar time frame is needed for fluorescence in situ hybridisation (FISH). Recording images takes time. The time which is needed for collecting an image series is dependent upon the number of sections, necessity for averaging, resolution in pixels, simultaneous or sequential scanning mode. The user should be aware of these constraints if many samples have to be examined within 1 day.

Image analysis is usually done at a later stage, most often using a different computer in order to have the LSM instrument available for recording data. For quick visualisation, it is a good idea to produce, e.g. a maximum intensity projection (MIP) which results in a 2D image of the image series (*see* **Note 9**). The MIP is usually good enough if every single section contains limited information. The MIP looks overloaded if every single section contains a lot of signal/information. That does not mean the data set has low quality, but in this case, other modes for projection may be better choices such as XYZ projection, 3D orthogonal view, 3D volume view, 3D isosurface view. Projecting images in 3D with a variety of tools and options using a sophisticated program requires more time in order to achieve good results. Quantification – programs which only count pixels (2D) or voxels (3D) work fast (seconds). However, programs recognising objects need a much longer time for calculation (hours).

3.10 Troubleshooting

1. Low signal
   (a) Staining OK?
   (b) Coverslip clean?
   (c) Front lens of objective lens clean?
   (d) Air bubbles in front of lens?
2. No signal
   (a) Microscope settings OK?
   (b) Laser settings OK?
   (c) Check focus and staining using visual epifluorescence or transmitted light.
   (d) Control if laser is visible, light path OK, excitation wavelength right and emission detection setting matching fluorochrome.

(e) Double check settings at microscope (mechanical in older types) and in software.

3. Preventive maintenance
   (a) Check the alignment of the system periodically (e.g. using focal check fluorescent beads).
   (b) Use reference samples and reference images to assess instrument performance.
   (c) Check accuracy of stage movement using fluorescent beads of known size.
   (d) Have periodic (annual) alignment by the manufacturer.

4 Research Needs

Given the tremendous advances in laser microscopy imaging over the last 20 years, it is somewhat surprising that so few studies have applied the different LSM approaches in hydrocarbon research. In general, there is substantial opportunity to carryout microscopy-based studies of microbial interactions with hydrocarbons including:

- Expanded application of fluorescent probes to cover a range of parameters including the micro-environmental conditions associated with hydrocarbon degradation
- Further use of time-course studies of events including the degradation of the hydrocarbon
- Greater attention to calibration of imaging and quantification of events
- Ground truth of events associated with microbial colonisation and transformation of hydrocarbons
- Use of correlative microscopy where possible to study events

5 Notes

1. Spacers of different but defined height can be cut from glass slides, coverslips, plastic material, thin plastic sheets, O-rings, etc.. Spacers may be also purchased from microscopy equipment suppliers.
2. Depending on the mounting procedure and microscopy supplies used, floating of the hydrocarbon droplet at the water surface may be critical in terms of the working distance (inverted microscope). Another issue is sorption of the liquid hydrocarbon to, e.g. the coverslip or spacers, resulting in either deformation of the droplet or even loss during placement of the coverslip (upright microscope). It may also be useful to have a

temperature-controlled stage or use warm or cold packs on the stage to regulate temperature for bacterial growth and fluidity of the hydrocarbon.

3. Control – make sure that the unstained sample is checked for possible autofluorescence. In certain samples, a specific autofluorescence can be used for imaging. This maybe the case for cells, e.g. cyanobacteria or algae, as well as for specific hydrocarbons having ring structures.
4. Apart from Nile Red, there are many other lipophilic fluorochromes available, e.g. DiI-C_{18}, DiO-C_{18} or membrane-specific ones, e.g. FM1-43 and FM4-64. They have different HLB values (hydrophilic–hydrophobic balance) as well as different excitation and emission characteristics.
5. Due to sample properties and potential quenching effects, it is a good idea to check other nucleic acid-specific fluorochromes such as SYBR Green.
6. Most important is the proper selection of the objective lens. There are two contradictory issues to consider: resolution and working distance. Resolution is determined by the numerical aperture (NA) of the lens and not by the magnification! The magnification only determines the area you look at. However, high NA means short working distance or vice versa; long working distance means low NA. Nevertheless, for samples extending into axial direction (thick samples or objects on a 3D topography), lenses with a long working distance are necessary. An ideal solution is using water immersion or water-immersible lenses. They are available in two forms: (1) corrected for a coverslip or not corrected for a coverslip (both with high NA) and (2) as long working distance objective lens to be used without a coverslip (low NA). All objective lenses produce bright images and have been used for many different sample types.
7. Always write a protocol and take notes of all the settings used. For this purpose, a spreadsheet is quite useful. In the future, it will allow one to quickly look up the details without going back to the microscope which is usually occupied by someone else, and there is no need of time-consuming searching and loading of the images. The spreadsheet will allow listing of sample preparation and details of the settings used for each image series. The main points are mounting, staining, laser lines, filters, magnification, resolution, pixel/voxel size, thicknesses, number of images, step size, zoom, average, channels, PMT voltage, image number *and most important comments*. These details are used later to judge image quality, assess suitability of images for specific projections, select images for publication, ensure proper scaling and comprehend what and why things were done in that particular way.

8. It is very important in digital image analysis to be aware of (1) what has been seen visually in the (epifluorescence) microscope attached to the LSM, (2) what has been recorded as raw data (on screen) and (3) what changes were applied to the raw data during visualisation. Always apply changes to a copy of the data set, and always compare changes with what has been seen in the microscope. Please consult the following publications on critical issues in scientific imaging [38].
9. The MIP represents only an intermediate result! In fact, it is the image to be remembered. It has a small file size and can be carried away on a USB stick. The MIP may then serve together with the laser microscopy protocol as a basis for discussion and subsequent image analysis. The latter of course is done with the original raw image data set (3D series).

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