18

Reflection Contrast Microscopy

The Bridge Between Light and Electron Microscopy

F. A. Prins, I. Cornelese-ten Velde, and E. de Heer

Summary

Reflection contrast microscopy (RCM) is a light microscopic method to image cells at high definition and enhanced sensitivity compared to conventional bright-field microscopy. RCM images have very high contrast, which makes them easily applicable for digital image analysis. Because ultrathin sections are mostly used in this method, RCM also functions by bridging light with electron microscopy: the combination of ultrastructural with histochemical studies. RCM can also replace electron microscopy for rapid and simple screening of large quantities of samples for immunocytochemical staining. Special attention is paid to small biological objects, which have to be processed for RCM. If you encounter the limits of brightfield microscopy, in resolution, sensitivity or handling of the specimen, RCM will be a feasible option.

Reflection contrast microscopy methods use only slightly adjusted electron microscopy methods for specimen preparation. Therefore, many familiar techniques for ultrathin specimen preparation can be applied. It is essential that only refractive index differences exist in those areas that are of interest and that the further specimen is as optically homogenic as possible, with a refractive index as close to that of glass as possible. Therefore, plastic embedding is recommended.

Key Words: Ultrathin section; immunocytochemistry; high definition; specimen preparation; reflection mode of CLSM; reflection contrast microscopy; enhanced detection sensitivity; image analysis; bright-field microscopy; transmission electron microscopy; high-resolution; high-contrast microscopy; combining light and electron microscopy.

1. Introduction

Reflection contrast microscopy (RCM) can be used as a bridge between light microscopy (LM) and electron microscopy (EM) because it visualizes ultrathin sections, normally used in transmission electron microscopy (TEM), at the light microscopic level (*see* **Fig. 1A**). The main advantages of RCM (properties) (compare **Fig. 1B–D**) are increased sensitivity and image definition compared to bright-field microscopy; the theoretical resolution (200 nm) can actually be

From: *Methods in Molecular Biology, vol. 319: Cell Imaging Techniques: Methods and Protocols*
Edited by: D. J. Taatjes and B. T. Mossman © Humana Press Inc., Totowa, NJ

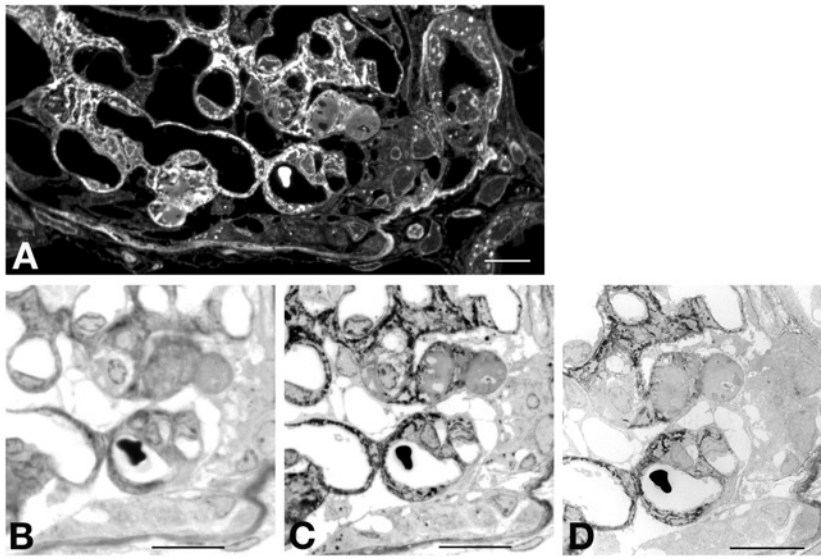

Fig. 1. High-power images of sequential sections of the distribution of laminin in glomeruli during development of glomerulosclerosis in mice with experimental lupus nephritis. These glomeruli demonstrate typical properties of RCM imaging: increased definition, enhanced contrast, and increased sensitivity in detecting the immunoperoxidase product of diaminobenzidine. **(A)** RCM image of ultrathin Epon-embedded section counterstained with toluidine blue. Using reflection, there is a larger overview of the whole section than by using TEM. **(B)** Bright-field microscopic (BFM) image of 1-µm-thick section counterstained with toluidine blue. **(C)** The same as **A**, but digitally inverted, for ease of comparison with BFM and TEM image. **(D)** TEM image of a sequential ultrathin section, which shows the detection of the DAB-ox with high resolution (only lightly contrasted with lead citrate). Bar = 20 µm.

obtained in RCM. Compared to TEM, RCM provides fast information at an overview. For instance, immunolabel can be detected at such a low magnification (630–1000×) that it facilitates finding the labeled sites in TEM to study its ultrastructure *(1,2)*. RCM is a suitable method for even the tiniest objects (morphological study using a simple light microscope), for which you initially do not know anything else than to subject them to TEM analysis (*see* **Note 1**), or to perform immunofluorescence (e.g., leptospires in **ref.** *3*). With RCM, one can make images of small to extremely small objects, biopsies, and single cells without the need for any immunocytochemical staining because you see all there is, which have a refractive index different from glass. In **Tables 1** and **2** many types of application and discipline are listed in which RCM has been used. A special feature of RCM technology is the use of sequential ultrathin sections, so that almost the same tissue can be studied by RCM and TEM; the distance between the sections is 50–100 nm (*see* **Fig. 1C,D**).

Table 1
Type of RCM Applications Using RCM

Immunolocalization studies at (sub) cellular level in cells and tissues Comparison of sequential ultrathin sections for multiple labels
In situ hybridization
Autoradiographic detection
Tracer studies of molecules conjugated with a label (radioactive, peroxidase [PO], gold, or indirect with a labeled antibody)
Detection of small inclusions, fragmented foreign material (different refractive index)
Detection at LM level of objects below the resolution of the LM, as a result of a bright, isolated signal against a dark background
Previewing of electron microscopic ultrathin sectioning of tiny or thin objects
Quality control of cell and tissue culture
Attachment and movement of living cells

Table 2
Type of Discipline Using RCM

Discipline	Method	Resin	Label	Ref.
Nephrology	Post-embedding; Pre-embedding	Lowicryl K4M; EPON	IGS/IGSS, Po-DAB	***17–20***
Oncology	Pre-embedding	EPON	Po-DAB	***15,21,22***
Pathology	Pre-embedding	EPON	Po-DAB	***3,18,22***
Cell biology	Post-embedding; living cells	Lowicryl K4M	IGSS	***7,8,15,23–25***
Pharmacology	Auto radiography	Spurr	Silver	***26***
Neurology	Post-embedding	Spurr	IGSS	***1,2,5,6,27***
Plant biology			IGSS	***28***
Transplantation	Post-embedding	Lowicryl K4M	IGS/IGSS	***29***
Molecular biology	*In situ* hybridization		APh/Po-DAB	***16,30–32***
Overview of study's RCM				***3,6,7,11,33–40***

Abbreviations: IGS, immunogold staining; IGSS, immunogold silver staining; Po-DAB, peroxidase–diaminobenzidine; Aph, alkaline phosphatase.

All types of TEM specimen preparation can be used for RCM, so that the book by Nasser Hajibagheri on electron microscopy is very useful ***(4)***. The main modification for RCM use is that ultrathin sections are placed on glass slides. Also,

less rigorous fixation can be used, omitting osmium tetroxide (which influences images formation because of strong reflection) or sometimes glutaraldehyde to maintain antigenicity in case of immunocytochemistry. Fewer specimens are useful for RCM from the LM specimen preparation field—only plastic-embedded and whole-cell (chromosomes, etc.) preparations. Generally, the LM section is too thick, which yields blurred images. However, optically quite homogenic objects with a strong reflecting label (like silver-stained neurons) can be used with RCM to visualize the label in depth through the object ***(5,6)***. Thus, with good understanding of the image formation in RCM (*see* **Subheading 1.1.**) many new objects, specimens, and applications can be discovered or developed.

Living cells also can be studied by RCM. Actually, this was the first application of RCM ***(7)***. In an aqueous environment, cell contacts with the glass are less reflective (darker) and can be studied ***(8)***.

1.1. Principles of Reflection Contrast Microscopy

Reflection contrast microscopy is a light microscopic technique where image formation is based on reflected light rays. It uses a microscope with incident light, in contrast to transmitted light, which is used for bright-field microscopy. The RCM images look like fluorescence images, because generally the background is dark (*see* **Note 2**). They are also made on an adapted fluorescence microscope, which is equipped with devices so that only the light reflected at the object forms the image. This is extremely difficult for biological objects, for which the reflected light intensities are generally much less than 1% of the incident light, which is very low compared to the 4% reflection intensity at a glass–air interface (of which there are many in a microscope); therefore, an optical trick is applied so that only the light reflected at the object forms the image. This omittance of disturbing reflections, which would decrease contrast in the image, is indicated by the additive "contrast" in RCM.

The microscope conformation of RCM consists of a combination of two contrast-enhancing principles: the antiflex principle ***(9)*** and the central stop system ***(10)***. The latter is located in front of the lamp house to create access to an aperture plane conjugate with the upper entrance pupil of the objective, and it consists of central black circles and aperture diaphragms (the size depends on the objective lens). Passing this combination makes the light beam ring shaped. The central stop prevents the central part of the light beam from reflecting at the backside of the objective (in a RCM, it prevents the existence of rays, which otherwise would reflect against the underside of the glass slide, (the amount of light at this glass–air interface is about 4%) and blurs the object image (*see* **Note 3**). The antiflex principle consists of a set of crossed polarizers (a polblock) and a quarter-wavelength plate (located at the front lens of the objective). It functions as follows. The light is linearly polarized by a 0° polarizer,

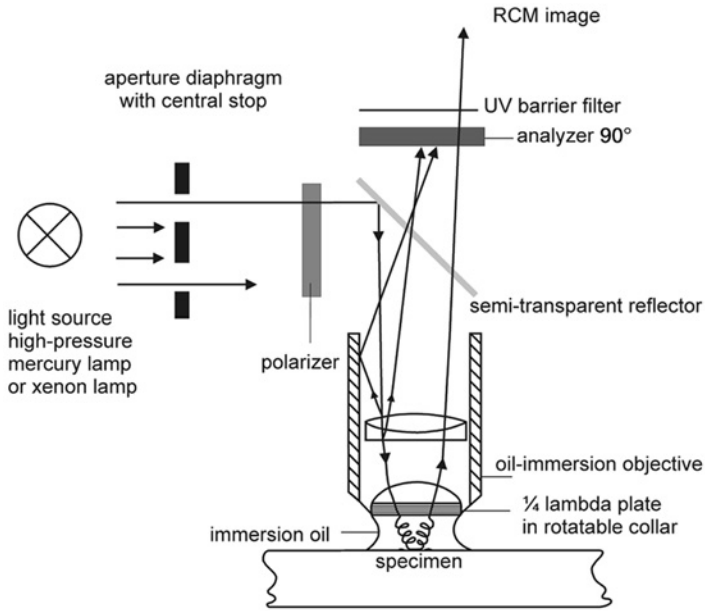

Fig. 2. Light path of the RCM.

deflected by the 50/50 reflector in the vertical light path, and upon passing the quarter-wavelength plate, the light is circularly polarized. After reflection at the object, the light passes the quarter-wavelength plate again and is rotated another 45°. Related to the incident ray, the polarization state has turned 90°, so that this light (reflected at the object) can pass the analyzer, which is crossed to the polarizer. All light rays that reflect inside the microscope are stopped by the analyzer. The light path is depicted schematically in **Fig. 2**.

Image formation in RCM is based on both reflection and interference of reflected light rays ***(11)***. What we see of our world is based on reflection of light. Oil on a water surface is seen as colors because of interference of the reflected light waves at the surface oil–air interface with those reflected light waves at the oil–water interface. The colors seen also depend on the thickness of the oil layer and on the angle of reflection. Translated to a microscope image, the thickness of an object, the numerical aperture (NA) of the objective, and the size of the aperture diaphragm (outer ring of central stop) determine the image formed next to the properties of the object (i.e., the refractive index, absorption coefficient, and surface texture). Reflection of light occurs at an interface of refractive index differences, and the intensity of reflection is proportional to the difference in refractive indices. Interference of reflected light occurs in thin layers, where, at the first interface, a portion of the light ray is reflected and the

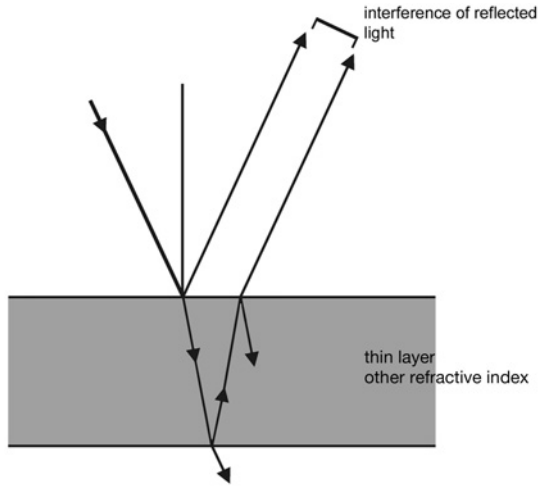

Fig. 3. Principle of interference of reflected light waves in thin layers.

remainder is transmitted; this transmitted light ray reflects at the next interface, transmits the former interface, and can interfere with the directly reflected part of its ray because they have the same frequency (*see* **Fig. 3**).

1.2. Introduction to the Methods

The methods are given for the whole procedure from tissue to RCM image. This is a very large field and many books even in this series have been written about this. Thus, we present our main routine for the preparation of specimens. Moreover, methods in the TEM field for preparing ultrathin sections can be used, including immunocytochemistry, with pre-embedding and postembedding labeling, different plastics, ultracryomicrotomy, and so forth. Methods for specimen preparation from the fields of parasitology, microbiology, or cytogenetics can also be useful.

Because ultrathin sections are needed (*see* **Note 4**), in general the tissue is cut in small pieces (2 mm^2) or one starts with smaller objects, which are fixed (lightly for maintaining antigenicity in case of immunocytochemistry) and embedded. Two methods are given: one for the morphological study and the other for immunocytochemical study. For immunocytochemical staining, there are two possibilities: either the immuno-incubation is performed before (pre-) or after (post-) embedding of the tissue. In pre-embedding immunocytochemistry, the tissue is mostly sliced with a vibratome in sections of about 70–90 µm thickness, which are immunostained, followed by embedding in plastic and cut in ultrathin sections. In postembedding immunocytochemistry, the ultrathin sections are stained, and depending on the type of embedding media used

(affecting accessibility of the antigenic sites for the antibodies), further etching steps might be needed (e.g., EPON™). However, whole cells, cultures, suspensions or micro-organisms, chromosomes, or other isolated cell-constituent specimens can be made, with or without embedding and ultrathin sectioning. Even living cells can be studied (*see* **Subheading 3.2.**).

Reflection contrast microscopy can be easily performed if a fluorescence microscope stand is available. The adaptation is provided in a method, followed by operating the RCM. If a confocal laser scanning microscope is available, we will describe how to make RCM images with it. In the last part, some methods about recording and interpretation of RCM images are presented.

2. Materials

2.1. Specimen Preparation

2.1.1. Major Equipment and Supplies

1. Razor blade (single edge), wax plate, pair of tweezers, rubber policeman.
2. Stereo microscope with light source.
3. Ultramicrotome (Leica UCT) or a routine, motorized microtome (Leica RM2165).
4. Vibrating blade microtome (Leica VT 1000 S).
5. Small freezer with top lid (–20°/–30°C).
6. Eppendorf capsules, 1.5 mL.
7. Flat embedding molds (Agar Scientific Ltd., UK, cat. no. G3531).
8. Small Beem capsules (size 3 and 00) (Polaron, England).
9. Beem capsules with conical tip (Agar Scientific Ltd., UK, cat. no. G361-1).
10. Glass, cryo-, or regular diamond knife (Diatome-Switzerland or Drukker, The Netherlands) (*see* **Fig. 4**).
11. Small wire loop (3 mm in diameter) (Agar Scientific Ltd., UK, cat. no. T5011) (*see* **Fig. 4**).
12. Coated glass slides (*see* **Subheading 3.1.1.**).
13. Transwell filters (Costar, Cambridge, UK, cat. no. 3414).
14. Immunofluorescence slides, 10 wells (Polysciences Europe, cat. no.18357).
15. Hot plate (30–80°C).
16. Ultraviolet polymerization lamp (Polysciences Europe, cat. no. 8778).
17. Moist chamber.
18. Dako pen (cat. no. S 2002) and a water-resistant ink pen.
19. Glass writing diamond marker (A. J. Cope Ltd., UK, code LA530).
20. Polyethylene gloves.
21. Burner for ethanol (*see* **Fig. 4**).

2.1.2. Major Chemicals and Solutions

1. Hank's culture wash medium.
2. Phosphate buffer: Solution A: 0.2 *M* sodium di-hydrogen ortho-phosphate (NaH_2PO_4), 0.497 g $NaH_2PO_4 \cdot 2H_2O$ in each 100 mL. Solution B: 0.2 *M* di-sodium

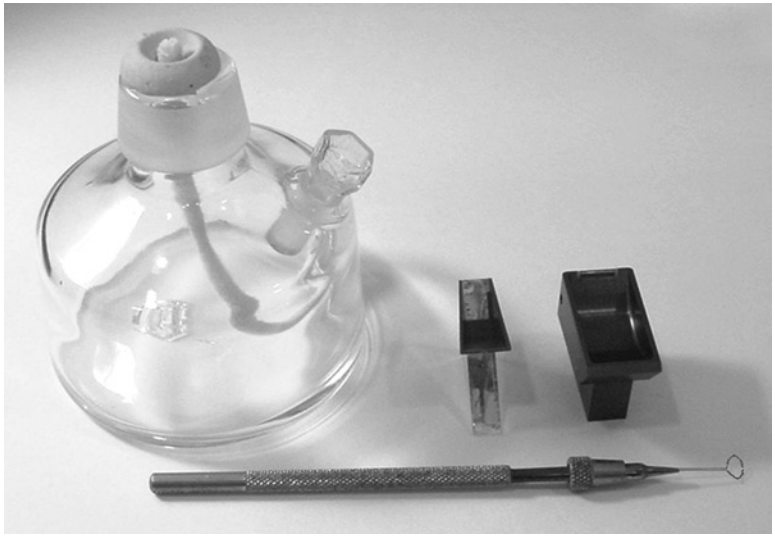

Fig. 4. Tools for ultrathin sectioning. Left to right: burner for ethanol, glass knife, diamond knife, and wire loop.

hydrogen ortho-phosphate (Na_2HPO_4), 2.328 g Na_2HPO_4 in 100 mL.To prepare 0.2 *M* phosphate buffer, pH 7.4, mix 20 mL solution A and 80 mL solution B.

3. 0.2 *M* Sodium cacodylate buffer: 21.4 g sodium cacodylate in 400 mL distilled water, adjust pH to 7.4 with 1 *M* HCl and fill up to final volume of 500 mL (storage: at 4°C; stability: a few weeks; hazards: sodium cacodylate is toxic by inhalation, ingestion, and skin contact; irritation to skin, eyes, and mucous membranes; inhalation of small concentrations over long period will cause poisoning and should be treated as a suspected carcinogen; and could cause adverse mutagenic or teratogenic effects; toxicity data: LD_{50} = 2600 mg/kg oral, rat).
4. Store buffer morphology: mix 0.2 *M* sodium cacodylate buffer with the same volume of distilled water (1:1) and add 3% sucrose.
5. Store buffer immunohistochemistry: 2% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4.
6. Phosphate-buffered saline (PBS): 150 m*M* NaCl, 10 m*M* phosphate buffer, pH 7.4.
7. Incubation buffer: 1% bovine serum albumin (BSA), (Sigma, cat. no. A-9647), 0.1% gelatin (Merck, cat. no. 070), in PBS, mix together on a hot plate at 60°C.
8. 2% BSA in PBS (put 100 mL PBS in a glass vial and add 2 g BSA on top of it; do not stir until it is dissolved).
9. 0.2% Aurion BSA-C in PBS (Aurion, The Netherlands).
10. Aldehyde quench buffer: 0.05 *M* glycine in PBS, or 0.5 *M* ammonium chloride in PBS.
11. Sodium azide.

12. Protein blocking buffer: Add 5% normal serum (same species as secondary antibody) to PBS.
13. DAB: freshly prepared 0.05% 3,3´-diaminobenzidine tetra-hydrochloride (Fluka) in 0.05 *M* Tris- HCl, pH 6.0.
14. 30% Hydrogen peroxide (Merck, cat. no. 7209). *Note:* hazard.
15. 0.2% Light green in distilled water (BAH/Gurr, cat. no. 34204).
16. 0.2% Mayer's hematoxylin (Merck, cat. no. 15938) in distilled water.
17. 1% Toluidine blue in distilled water.
18. Aminosilane glass-coating solution: 2% Amino-propylethoxylane (Sigma, cat. no. A-3648) in acetone (storage: at 2–8°C; hazards: corrosive, causes burns; harmful by inhalation, in contact with skin, and if swallowed; wear suitable protective clothing, gloves, and eye and face protection).
19. Paraformaldehyde (Merck, cat. no. 4005) (storage as a solution at 4°C; stability: best to prepare fresh for each fixation, but can be stored for a few weeks; hazards: paraformaldehyde harmful by ingestion and irritant to the skin, eyes, and respiratory system; toxicity data: LD_{50} = 800 mg/kg oral, rat; LC_{50} = 0.9 mg/L inhalation, rat; should be handled in a fume hood with gloves).
20. 25% Glutaraldehyde (BDH, cat. no. 360802F): "EM" grade should be used (storage at 4°C; stability: several years; hazards: harmful by ingestion and inhalation; extremely irritating to the eyes; no prolonged skin contact; toxicity data: LD_{50} = 134 mg/kg oral, rat. Should be handled in a fume hood with gloves).
21. Osmium tetroxide (BDH, cat. no. 36219). (Should be handled in a fume hood with gloves.)
22. Lowicryl K4M kit (PolySciences Ltd.). (Storage: at 25°C; hazards: harmful by ingestion and inhalation; irritating to skin; eyes, and respiratory system. Should be handled in a fume hood with gloves).
23. Unicryl (Storage: at 4°C; hazards: harmful by ingestion and inhalation; irritating to skin, eyes and respiratory system; toxicity data: LD_{50} = 5000 mg/kg oral, rat. Should be handled in a fume hood with gloves).
24. Epon (Poly/Bed 812 kit 21959, Polysciences, Germany). *Note:* hazard.
25. Spurr's resin (resin kit R1032, AGAR Scientific, UK) *Note:* hazard.
26. Immunogold reagents: Either pA-, pG-, or IgG-bound gold particles (size 1–40 nm) (Amersham, UK or Aurion, The Netherlands).
27. Primary antibodies.
28. Rapid mounting medium ("Entellan"; Merck, cat. no. 7961).
29. Immersion oil for fluorescence microscopy; refractory index (n_e) = 1.518 (23°C) (Zeiss).
30. 1% Periodic acid in distilled water.
31. 10% Gelatin in PBS.

2.1.3. Glass Slide, Cleaning, and Coating

1. 96% Ethanol.
2. Acetone (Pre analyze [PA] quality).

3. Aminosilane solution: 2% aminopropylethoxylane (Sigma, cat. no. A-3648) in acetone.
4. Glass slides, all types of ready-to-use microscope slides.

2.1.4. Fixatives

1. For immunohistochemistry: 4% paraformaldehyde in 0.1 *M* phosphate buffer. Dissolve 8 g of paraformaldehyde in 100 mL distilled water by heating to 60°C. Add a few drops of 1 *N* sodium hydroxide until the solution clears, cool to room temperature, and filter. Mix 10 mL of 8% paraformaldehyde with 10 mL of 0.2 *M* phosphate buffer and add glutaraldehyde (25%) to a final concentration of 0.05 to 0.2%.
2. For morphology: 1.5% glutaraldehyde and 1% paraformaldehyde in 0.1 *M* cacodylate buffer adjusted to pH 7.4 for the primary fixation (mix 6 mL of 25% glutaraldehyde, 12.5 mL of 8% paraformaldehyde, and 50 mL of 0.2 *M* cacodylate buffer, then add 31.5 mL of distilled water). If EM is also used, postfix with 1% osmium tetroxide in 0.1 *M* cacodylate (mix 2 mL of 2% aqueous osmium tetroxide with 2 mL of 0.2 *M* cacodylate buffer, pH 7.4).

2.1.5. Pre-Embedding

1. Vibrating blade microtome (Leica VT 1000 S).
2. Beem capsules (size 3 and 00) (Polaron, England).

2.1.6. Embedding Resins

1. Epon (Poly/Bed 812 kit, cat. no. 21959; Polysciences, Germany). Mix 45 mL nadic methyl anhydride (NMA), 50 mL dodecinyl succinic anhydride (DDSA), and 80 mL Poly/Bed 812. Divide into 10-mL portions in 20-mL glass vials and store in a freezer (several months). Before using, add 0.15 mL 2,4,6-{tri(dimethylaminoethyl)phenol} (DMP-30) to 10 mL Epon mix and stir well.
2. Spurr's resin (resin kit, R1032; AGAR scientific, UK). *Note:* hazard. Mix 52 mL nonenyl succinic anhydride (NSA), 20 mL vinyl cyclohexene dioxide (ERL 4206), 14 mL diglycidyl ether of polypropylene glycol (DER 736), 1.2 mL dimethylaminoethanol (DMAE) or equal amounts just before using.
3. Lowicryl K4M (resin Polar Kit, cat. no. 15923; Polysciences Inc., Germany). *Note:* hazard. Mix 2.25 g crosslinker (A) and 12.75 g monomer (B), and 0.075 g initiator (C).
4. Unicryl (resin kit, cat. no. BA250; BB International). *Note:* hazard.

2.1.7. Ultracryo-sectioning

1. Ultramicrotome equipped with cryo-attachment (Leica Germany).
2. Diamond cryoknife or a glass knife.
3. Copper or aluminum cryo-specimen mounting pins (Leica, Germany).
4. Small wire loop (3 mm in diameter) made from platinum wire (0.2 mm) and mounted on a wooden stick (self-made).
5. 2.3 *M* Sucrose in PBS.
6. Liquid nitrogen Thermos flask.
7. Liquid nitrogen storage tank (for specimen storage).
8. Aminosilane coated glass slides (*see* **Subheading 3.1.1.**).

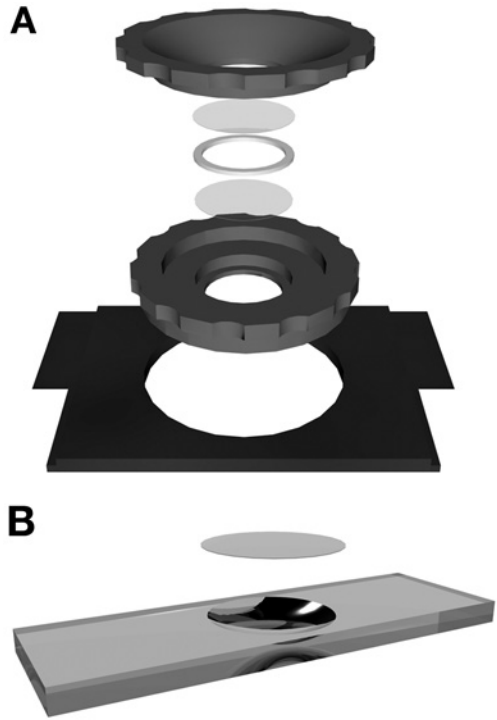

Fig. 5. Cell culture/observation chambers: **(A)** specially designed chamber; **(B)** glass slide with cavity.

2.2. Observation of Living Cells

1. Round cover glasses (25 mm in diameter) (Omnilabo International, The Netherlands; cat. no. 211045).
2. Glass slide with cavity (*see* **Fig. 5B**) (Omnilabo International, The Netherlands; cat. no. 408501).
3. Cell culture chambers (Agar Scientific Ltd., UK cat. no. G3541) or special chambers, with a silicon-ring between two round cover glasses (*see* **Fig. 5A**).
4. Nail polish.

2.3. RCM Adaptation Equipment

1. An incident light microscope, such as a fluorescence microscope, Leica DMR, or Zeiss Axioskop.
2. RCM oil-immersion objective equipped with a quarter lambda plate in the front lens system (*see* **Fig. 6D**): Plan-neofluor 63×/1.25 oil Antiflex from Zeiss, or the HCX PL Fluotar 100×s/1.30 oil RC from Leica.
3. A lamphouse with a high-pressure mercury or xenon lamp as used in fluorescence microscopy.

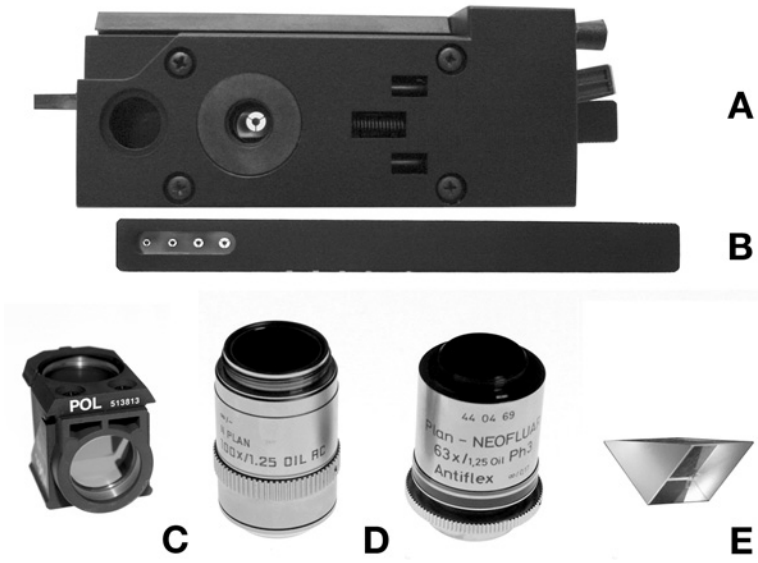

Fig. 6. Equipment for adaptation of an incident/fluorescence microscope: **(A)** RCM module containing, the following: **(B)** slider with different central stops, **(C)** POL-block, **(D)** RCM objectives, and **(E)** glass prism.

4. A RCM module, Leica (*see* **Fig. 6A**) adapting one slider with different sets of central stops (*see* **Fig. 6B**).
5. A POL- block containing a polarizer, a neutral beam splitter, and an analyzer (*see* **Fig. 6C**), a 10-mm Right-Angle Prism (Melles Griot, code: 01PRB 009 http://www.mellesgriot.com) (*see* **Fig. 6E**), instead of the central stop.
6. Protection filter (preventing heat, infrared) for the polarizer.

2.4. Confocal Laser Scanning Microscope in the Reflection Mode

1. Confocal laser scanning microscope (LSM-510, AxioPlan-2 from Zeiss, Jena Germany, or TCS SP2, DM-I RBE from Leica, Heidelberg, Germany). An inverted or upright microscope can be used. Laser lines: argon (range: 450–514 nm; 200 mW); helium–neon (He–Ne1; 543 nm; 4 mW); helium–neon (He–Ne2; 633 nm; 15 mW).
2. Important components for confocal RCM are oil-immersion objectives and a neutral beam splitter (always present in a confocal laser scanning microscope [CLSM]).
3. 5× or 10× Dry objective.

2.5. Recording of RCM Images

1. Conventional analog camera in combination with Dia positive film of 100 to 200 ASA.

2. Examples of digital cameras are Leica DC 200, and DC 300 and the AxioCam from Zeiss.

3. Methods

3.1. Specimen Preparation From Tissues and Cells

The protocol is as follows:

- Fixation of tissue/cells to a small cubic form;
- Pre-embedding immunostaining*;
- Dehydration and embedding of blocks;
- Sectioning of blocks;
- Ultracryosectioning*;
- Staining of sections (immuno)-histochemistry.

Very thin objects yield RCM images with the highest image definition. To visualize or immunolabel the inside of a cell (intracellular labeling), it has to be sectioned. To section tissue/cells ultrathin, they must be made firm by embedding in plastic or by freezing. Before infiltration with the plastic or freezing, tissue/cells need to be fixed: with a mild fixative for immunocytochemical studies so that antigenicity is maintained, and with a strong fixative for a morphological study (*see* **Note 5**). Ultrathin sectioning (50–250 nm) of the embedded or frozen tissue/cells is then performed using an ultra-/motorized conventional ultramicrotome or an ultracryomicrotome. The sections are put on cleaned and coated glass slides and marked by a circle at the underside of the glass slide (for ease of finding the sections). Thereafter, the ultrathin sections can be immunostained (postembedding). Depending on the accessibility of the antigenic sites, further etching steps might be required (e.g., Epon and Spurr's). Ultrathin cryosections have the highest accessibility, followed by hydrophilic resins such as LR-White, Lowicryl, and Unicryl, in which the antigenic sites are accessible in the top layer, and, finally, Epon and Spurr's, which are least accessible. The cryo-method is the best option for studies with new antibodies, of which antigenic sites are unknown or the preservation of antigenicity upon fixation is unknown.

For inter-(extra)cellular labeling, or for instance extracellular matrix components in tissue, the pre-embedding method can also be chosen. A strong fixation is applied after the immunostaining step. This extra fixation is often applied in RCM methods.

Staining of antigens at the surface of (single) cells grown on glass can be visualized directly (i.e., without embedding and sectioning) with RCM or confocal laser scanning (CLS-RCM). However, when, for instance, internalization

*This step also comprises other steps from this protocol.

studies are performed with probes conjugated to reflective markers (colloidal gold, peroxidase), then in order to study the inside of the cells, they have to be sectioned and the appropriate method is preembedding.

For single-cell/small biopsy studies with these RCM methods, there are many ways to section the cells (e.g., parallel or perpendicular to the attachment substrate of the cells). Another advantage of the method is that because of the thickness (50–100 nm) of ultrathin sections, many sections can be made of even single cells. They can also be collected in their sequential order. Sequential (serial) sections can be collected consecutively on glass slides, or one on glass and the next on grid and respectively be used for localization of different antigens on sequential sections (multilabeling studies) or in combination with EM (subcellular localization).

A central theme in methods for preparing RCM specimens is to adhere to clean working conditions; because of the sensitivity of RCM, any dirt or debris is visible. Therefore, all glass slides used must be cleaned, and some solutions millipore-filtered (*see* **Note 6).** For immunohistochemistry, coated slides are used.

3.1.1. Glass Slides, Cleaning, and Coating

For morphological studies:

1. Clean slides by washing them with 96% ethanol for 1 min and with acetone for 1 min.
2. Wipe dry with tissue in one movement (avoid dust) and store in a dust-free box.

For immunostaining:

1. Wash slides with acetone for 2X 10 min.
2. Coat overnight with 2% aminosilane in acetone in a covered jar.
3. Wash with acetone for 2X 10 min.
4. Wash with distilled water for 5 min.
5. Wipe dry with tissue in one movement (avoid dust) and store in a dust-free box.

Alternatively, use glass slides with adhesive coating (Star-Frost Adhäsiv-Objektträger; Knittel-Gläser, Germany).

3.1.2. Fixation of Tissue/Cells to a Small Cubic Form

3.1.2.1. Tissues, Biopsies

After removal of the tissue, cut into small cubes (size <2 mm^3) on a wax plate with a sharp, new, single-edged razor blade and submerge the cubes in the appropriate fixative (*see* **Subheading 2.1.2.**) (*see* **Note 7**).

The fixation time for tissue should be at least 4 h at 4°C. After sufficient fixation, tissues or cells can be stored in the following: 3% Sucrose in cacodylate buffer 4 h or overnight at 4°C for morphology processing; 2% Paraformaldehyde/PBS 4 h or overnight at 4°C for immunoprocessing.

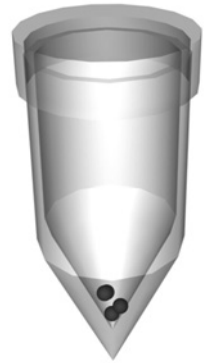

Fig. 7. Beem capsule with a conical tip with small samples.

3.1.2.2. Small Biopsies

Biopsies smaller than 2 mm^3 include spheroids, cell aggregates, needle biopsies, isolated glomeruli, or islets of Langerhans.

1. Fixation in Eppendorf capsules with conical tips (*see* **Note 7**).
2. Wash biopsies with wash buffer (do not centrifuge); carefully remove buffer after allowing the tissue pieces to settle by gravity.

For morphology, fixation is with osmium tetroxide (*see* **Note 8**); further processing (*see* **Subheading 3.1.4.**) is performed in Beem capsules with conical tips (*see* **Fig. 7**).

For immunohistochemistry, blocks are made of biopsies in gelatin:

3. Add 100 µL of 10% prewarmed gelatin/PBS (37°C) and mix.
4. Centrifuge quickly when gelatin is still liquid at 825*g* for 5 min.
5. Allow the gelatin to solidify in a refrigerator at 4°C for 10 min.
6. Cut off the bottom of the tube with a sharp, new, single-edged razor blade just above and beneath the gelatin. Also, carefully remove the surrounding remains of the tube.
7. The gelatin block(s) are postfixed in stored buffer for immunohistochemistry, at 4°C for 4–16 h.
8. Cut away any gelatin not containing cells and divide in cubes of 1–2 mm^3 under a stereomicroscope on a wax plate with a razor blade.
9. Process the pieces as described in the embedding procedure for tissue (*see* **Subheading 3.1.4.2.**) or ultrathin cryosectioning (*see* **Subheading 3.1.5.2.**, **step 3**).

3.1.2.3. Single-Cell Suspensions

1. Harvest cells after trypsinization and wash in culture medium with serum in 10-mL tube.
2. Centrifuge at 300*g* for 5 min.

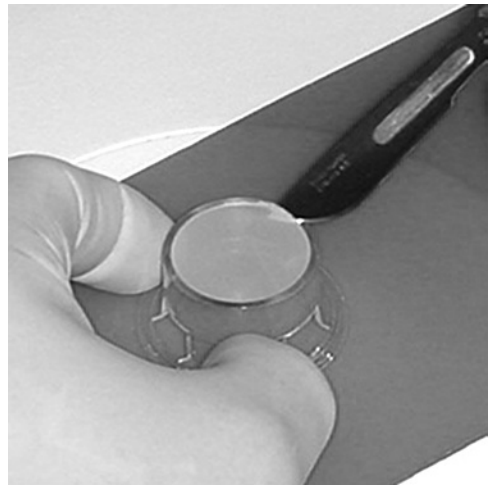

Fig. 8. Cutting away of the transwell filter.

3. Remove medium and dissolve pellet in fixation fluid.
   - For morphology, fix in 1.5% glutaraldehyde for 30 min at room temperature;
   - For immunohistochemistry, fix in 4% paraformaldehyde with 0.05–0.2% glutaraldehyde 30 min at room temperature (*see* **Note 7**).
4. Centrifuge at 300*g* for 5 min.
5. Remove the fixative and add 1 mL of PBS to the pellet.
6. Put the cells in an Eppendorf tube (1.5 mL).
7. Centrifuge again, remove the PBS, and gently loosen the pellet by tapping the tube. For further steps, see **Subheading 3.1.2.2.**, after **step 2**.

3.1.2.4. Monolayers (on Plastic or Glass)

1. Wash the cells with Hank's medium.
2. Fix the cell layer for 30 min at 4°C (*see* **Note 7**).
3. Remove the cells from the layer with a rubber policeman and collect in a centrifuge tube.
4. Proceed from **Subheadings 3.1.2.3.** and **3.1.3.4.**

3.1.2.5. Cells Growing on Transwells

1. Wash the cells with Hank's medium.
2. Fix the cell layer for 30 min at 4°C (*see* **Note 7**).
3. Carefully remove the filter from the well with a razor blade under a stereomicroscope and put this on a wax plate, with the cells facing upward (*see* **Fig. 8**).
4. Carefully cut the filter in slices approx 2 mm in width and place two or three slices on top of each other in a rubber embedding mold or place them upright in an Eppendorf tube.

5. Add 10% preheated gelatin/PBS (37°C) into the module or tube.
6. Allow the gelatin to solidify in a refrigerator at 4°C, or on ice.
7. Remove from the mold or cut off the bottom of the tube with a sharp, new, single-edged razor blade just above and beneath the gelatine. Also, carefully remove the surrounding remains of the tube.
8. Fix the gelatin with filters with the stored buffer overnight, also at 4°C.
9. Process the pieces as in the embedding procedure for tissue, described in **Subheading 3.1.4.** or ultrathin cryosectioning (*see* **Subheading 3.1.5.2.**, **step 4**).

*3.1.3. Tissue Processing for Pre-Embedding (*See ***Note 8****)*

All steps, including the glutaraldehyde fixation, are performed at 4°C to avoid bacterial growth; thereafter, steps are performed at room temperature.

1. Fix tissue blocks (4–10 mm^3) briefly after perfusion or by immersion with fixative for 16 h (*see* **Note 7**).
2. Cut vibratome sections (70–100 µm thick) and transfer sections with a small paintbrush into PBS in a 10-mL glass vial.
3. Proceed with all further incubation steps by gently stirring on a rotor.
4. Incubate sections with quench buffer for 1 h (*see* **Note 9**).
5. Permeabilize with 0.05% Triton X-100 in PBS for 30 min (*see* **Note 10**).
6. Incubate with protein blocking buffer for 30 min.
7. Incubate with specific primary antibody (1–5 µg/mL) in 1% BSA with 20 m*M* NaN_3/PBS (*see* **Note 11**) for 16 h (*see* **Note 12**).
8. Wash with PBS 5X 10 min.
9. Incubate in peroxidase-conjugated antibody in 1% BSA/PBS for 2 h. or incubate in appropriate colloidal gold (1 nm or 5 nm)-conjugated reagent in 1% BSA/PBS for 2 h.
10. Wash with PBS for 5X 10 min.
11. Fix with 1% glutaraldehyde in PBS for 20 min at room temperature.
12. Wash with PBS for 3X 10 min at room temperature.
    a. For Peroxidase–DAB Staining. Develop with freshly prepared diaminobenzidine medium (add 10 µL of 30% H_2O_2 to the filtered DAB solution) for 20–40 min in the dark. Wash peroxidase–DAB-stained sections with PBS for 3X 20 min.
    b. For Immunogold Staining (*see* **Note 13**) Wash sections thoroughly with distilled water 4X 15 min. Develop gold with silver reagent in the dark with the Danscher method for 30 min to 1h at room temperature (*see* **Subheading 3.1.6.4.**). Wash thoroughly with distilled water 4X 15 min.
    c. For both staining procedures, continue with **step 13** (*see* **Note 14**).
13. Postfix with 0.5% osmium tetroxide in 0.1 *M* cacodylate buffer, pH 7.4, at room temperature for 20 min.
14. Further processing (*see* **Subheading 3.1.4.1.**, **step 2**) is performed in Beem capsules size 00 with the tip cut off. The vibratome sections must be embedded in the cover of the Beem capsules by placing the capsule upside down (*see* **Fig. 9**). It is then possible to cut sections parallel to the surface of the vibratome sections.

Fig. 9. Beem capsule with vibratome section embedded in plastic.

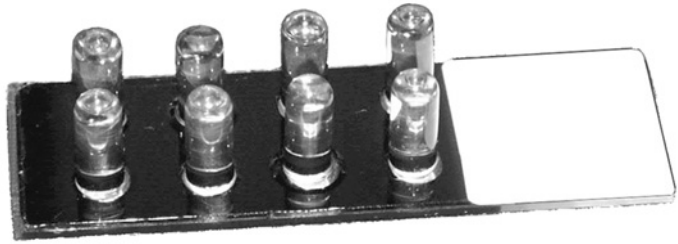

Fig. 10. Example of embedding in gelatin capsules of cells grown on glass.

3.1.4. Dehydration and Embedding

3.1.4.1. Dehydration and Embedding (of Tissue/Cells/Immunostained Vibratome Sections) in EPON

1. Postfix with 1% osmium tetroxide in cacodylate buffer (tissue for 1 h and cells for 10 min). If tissue must be studied by TEM and RCM, postfixation with osmium tetroxide is necessary (*see* **Note 14**). Wash with distilled water for 2X 10 min.
2. Dehydrate through an ethanol series, from 50%, 70%, 80%, 90%, and 2X 100% ethanol. All steps last 45 min for tissue samples and 5 min for single cells.
3. Embedding: remove the final absolute ethanol and add EPON/ethanol mix 1:1 overnight.
4. Infiltrate for 2 h with EPON or Spurr's/ethanol mix 2:1 followed by pure EPON or Spurr's resin for 2 h.
5. Finally, embed tissue blocks in Beem capsules or mold.

5a. For monolayer cells on glass or petri dishes (*see* **Note 15**), put EPON-filled gelatin capsules on top of them (*see* **Fig. 10**).

6. Polymerize the blocks at 60°C for 24–48 h.

Remove capsules with the embedded monolayer from the glass slide: Remove the excess EPON around the capsules with a small hand drill or with

a single-edged razor-blade. Dip the whole object glass in liquid nitrogen. Control the broken surface for glass particles under a stereomicroscope and remove them (*see* **Note 16**).

3.1.4.2. Dehydration and Embedding in Lowicryl K4M or Unicryl, for Immunohistochemistry

Cells and tissue samples are prepared for Lowicryl K4M or Unicryl resin embedding with minor modifications according to the progressive lowering-temperatures technique ***(12)***.

Dehydration:

1. Dehydrate the samples in 30% (v/v) ethanol at 4°C for 30 min.
2. 50% ethanol at –4°C for 30 min.
3. 70% ethanol at –20°C for 30 min.
4. 80% ethanol at –20°C for 30 min.
5. 90% ethanol at –20°C for 30 min.
6. 100% ethanol at –20°C for 30 min.

Infiltration:

7. Ethanol/resin (1:1) at –20°C for 60 min.
8. Ethanol/resin (1:2) at –20°C for 60 min.
9. Immerse in pure resin at –20°C for 60 min.
10. Immerse in pure resin at –20°C overnight.
11. Optionally, add fresh resin (60 min, –20°C) before final embedding of the samples in Beem capsules (*see* **Note 17**).

Polymerization:

12. Capsules are placed in a 96-well microtiter plate under ultraviolet (UV) lamps for 1 d at –20°C (*see* **Note 18**).
13. Polymerization is continued for 2 d at room temperature under UV lamps in a fume hood.

3.1.5. Sectioning

3.1.5.1. Sectioning for RCM and TEM, Collecting, and Storing

Sections can be cut on an ultramicrotome (100 nm) (*see* **Note 19**) or a routine motorized microtome (250 nm) using a glass or histo-diamond knife.

1. Clamp the specimen into the specimen holder and trim the excess resin from the block face and from the edges of the block using a single-edged razor blade.
2. Cut a semithin section of 1 µm, collect onto water, and use a preparation needle to transfer the section to a drop of water on a microscope slide.
3. Dry on a hot plate at approx 60°C and stain with toluidine blue for 1 min on a hot plate. Wash briefly with distilled water and dry on the hot plate for 20 min.

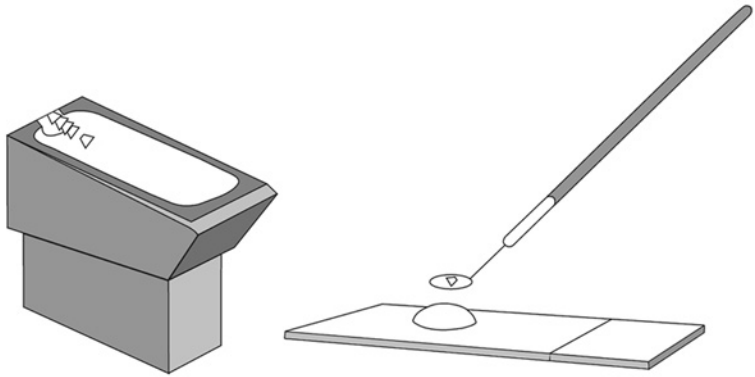

Fig. 11. Transfer of thin sections from a knife to a glass slide using a droplet in a wire loop.

4. Select an area using the microscope and trim the face to a trapezoid shape with a histo-diamond knife).
5. Fill the boat with water (*see* **Note 20**) and cut the ultrathin sections.

Collecting the Sections for TEM and RCM, Sequential Sectioning

For TEM: If you collect Lowicryl, Unicryl, or cryosections, use grids with a formvar or carbon film. For sequential TEM/RCM, one section from a small ribbon is picked up with a wire loop and the next one with a grid.

1. Cut a small ribbon; isolate the sections in pairs using an eyelash mounted on a cocktail stick with nail polish, which is also used for moving the sections on the water.
2. Separate the pair in single sections and place the grid in the water beneath one section and raise it at a slight angle so that the first section is in the center of the grid (*see* **Note 21**).
3. Slowly raise the grid out of the water and remove the water with filter paper; place the grid in a Petri dish with round filter paper to dry.
4. Move the wire loop into the water beneath the second section and raise it so that the second section is in the center of the loop; place it on a water drop on a clean glass slide (coated if immunocytochemistry will be performed) (under a dissecting microscope) (*see* **Fig. 11**). Clean wire loop after use in an ethanol flame (*see* **Fig. 4**).
5. Dry on a hot plate at approx 60°C for morphology and 40°C for immunohistochemistry.
6. Under a stereomicroscope, encircle the sections on the reverse side of the slide with water-resistant ink or, if a cap/prism will be used for microscopy, with a diamond writer (*see also* **Subheading 3.4.1., step 4**).

Only for morphology: Counterstain with 1% toluidine blue for 1 min on a hot plate and wash with distilled water and dry on a hot plate. Cover with a small

amount of normal mounting medium and use a clean cover glass. You can also use immersion oil as mounting medium; then fix the cover glass with nail polish. If you leave the section with oil without a cover glass, clean it with ethanol if it becomes dirty. Store slides in a dust-free slide container.

3.1.5.2. Ultrathin Cryosectioning for RCM, Starting From Fresh Material*

1. Fix fresh, living material with 4% paraformaldehyde and 0.1–0.2% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.4, for up to 2 h (*see* **Note 7**).
2. For cells; fix for 30 min and carefully scrape with a rubber policeman from the solid substrate, pellet by centrifugation in an Eppendorf tube (1.5 mL), and embed in gelatin as described in **Subheadings 3.1.2.2.** and **3.1.2.3**.
3. Cut sample into slabs and then into small blocks no larger than 2 mm^3 (*see* **Note 22**).
4. Transfer blocks into 2.3 *M* sucrose and leave overnight at 4°C (*see* **Note 23**).
5. Mount individual blocks onto specimen pins (supplied with cryo-ultramicrotome) and freeze by immersion in liquid nitrogen.
6. Cool cryo-chamber of ultramicrotome to –110°C and lock the knife into the holder.
7. Transfer the specimen pin to the cryo-chamber of the ultramicrotome.
8. Adjust thickness setting to section at 500 nm and cut into the block until a flat surface is produced.
9. Advance knife and specimen block and cut sections automatically to 100 or 150 nm.
10. Pick up sections on a drop of 2.3 *M* sucrose held in a wire loop (*see* **Fig. 11**).
11. Warm up the drop to which the sections are attached and place on a dry glass slide.
12. Store under sucrose at 4°C, or after marking around the drop of sucrose with a Dako pen, remove the sucrose with PBS and incubate directly for immunostaining (*see* **Subheading 3.1.6.1.** or **3.1.6.2.**).

3.1.6. Immunostaining

3.1.6.1. Immuno-Peroxidase Staining of Cryosections or Lowicryl/Unicryl Sections (*See* **Note 24**)

1. Perform all incubations in a moist chamber at room temperature.
2. Quench free aldehyde groups with quench buffer for 15 min (*see* **Note 9**).
3. Preincubate with protein blocking buffer for 10 min.
4. Incubate for 1 h at room temperature (or 16 h at 4°C) with specific antibody in incubation buffer.
5. Wash with PBS 4X 5 min in a jar.
6. Incubate with a peroxidase-conjugated secondary antibody in a suitable dilution in incubation buffer for 1 h.
7. Wash with PBS 4X 5 min.
8. Fix with 1% glutaraldehyde in PBS for 5 min (*see* **Note 25**).
9. Wash with PBS 2X 5 min.
10. Add 10 µL of H_2O_2 to 100 mL DAB solution. Develop in the dark with DAB + H_2O_2 solution for 10 min and wash with distilled water 2X 5 min.

11. Counterstain with light green for 2 min (*see* **Note 26**).
12. Wash with distilled water for 1 min.
13. Dry on a hot plate at 40°C for 10 min.
14. Cover the sections with a very small droplet of mounting medium and a clean cover glass.

3.1.6.2. Immunogold-Silver Staining of Cryosections or Lowicryl/Unicryl Sections

1. Start with **Subheading 3.1.6.1.**, **steps 1–5** (immuno-peroxidase staining); then preincubate with 0.2% solution of BSA-C in PBS for 10 min.
2. Incubate with a gold-conjugated (1–10 nm) secondary antibody in a suitable dilution in incubation buffer for 2 h.
3. Wash with PBS 4X 5 min.
4. Fix for 5 min with 2% glutaraldehyde in PBS.
5. Wash with PBS 2X 5 min.
6. Wash with distilled water for 4X 5 min.
7. Develop immunogold in the dark with the silver enhancer (mix just prior to incubation) for 20–40 min (*see* **Subheading 3.1.6.4.**).
8. Wash with distilled water for 4X 5 min.
9. Counterstain with toluidine blue for 2 min, with hematoxylin for 1 min, or with 1 μg/mL propidium iodide for 5 min (*see* **Note 26**).
10. Wash with distilled water for 1 min.
11. Dry on a hot plate at 40°C for 10 min.
12. Cover the sections with a small amount of mounting medium and a clean cover glass.

3.1.6.3. Immunogold-Silver Staining of EPON or Spurr's Sections

Immunoperoxidase can be applied on these sections as well.

1. Perform all incubations in a moist chamber at room temperature.
2. Etch with 10% H_2O_2 for 5 min; then wash with distilled water 2X 5 min.
3. Treat with 1% periodic acid solution for 10 min and wash with distilled water 2X 5 min.
4. Preincubate with protein blocking buffer for 10 min.
5. Incubate with specific antibody in PBS, 1% BSA, and 1% normal serum of second antibody for 1 h at room temperature (or 16 h at 4°C).
6. Wash with PBS for 5X 5 min.
7. Preincubate with 2% BSA in PBS for 10 min.
8. Incubate with a gold-conjugated (1–5 nm for RCM and 10 nm for TEM) secondary antibody in a suitable dilution in 2% BSA in PBS for 2 h.
9. Wash with PBS for 5X 5 min.
10. Fix with 2% glutaraldehyde in PBS for 5 min.
11. Wash with PBS for 2X 5 min, and wash with distilled water for 4X 5 min.
12. Develop immunogold in the dark with the silver enhancer (mix just prior to incubation for 20–40 min) (*see* **Subheading 3.1.6.4.**).

13. Wash with distilled water for 4X 5 min.
14. Counterstain EPON or Spurr's sections with toluidine blue for 2 min or Spurr's sections with hematoxylin for 1 min.
15. Wash with distilled water for 1 min.
16. Dry on a hot plate at 40°C for 10 min.
17. Cover the sections with a small amount of mounting medium and a clean cover glass.

3.1.6.4. Silver Enhancement of Gold ***(13)***

Only enhance with silver if the colloidal gold particles are smaller than 15 nm, because 15-nm gold particles are visible in RCM.

Solution A: Mix 25 g arabic gum in 50 mL distilled water for 1 d and filter through a gauze bandage. Dispense in 3-mL portions and store in freezer.
Solution B: Dissolve 25.5 g citric acid and 23.5 g sodium-citrate and check that pH = 3.5.
Solution C: Dissolve 0.85 g hydroquinone in 14 mL distilled water and filter through 0.2-µm filter. Prepare solution shortly (store for no more than 1 d) before use.
Solution D: Dissolve 0.11 g silver lactate in 14 mL distilled water and filter through 0.2-µm filter (store in the dark).

Before use, add 0.5 mL solution B, 0.75 mL solution C, and 0.75 mL solution D to 3.0 mL solution A, mix well, and drip with a Pasteur pipet on the sections and develop in the dark (e.g., incubation box or light-tight box). Make sure sections do not dry before the silver enhancement.

3.1.7. Single-Cell Experiments

3.1.7.1. Staining the Surface of Cells With Immunogold*

This is a nice specimen for demonstration of reflection with CLSM (*see* **Fig. 12**) (*see* **Note 27**). All steps were carried out at room temperature and incubation was performed in 24-well plates.

1. Culture cells on 10-mm-diameter cover glass in a Petri dish.
2. Wash with Hank's medium for 2X 1 min.
3. Fix with 1% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4, for 10 min.
4. Treat with aldehyde quench buffer for 20 min.
5. Preincubate with 2% BSA in PBS for 10 min.
6. Incubate with specific primary antibody diluted in 1% BSA in PBS for 1 h. Alternatively, use a colloidal gold (5–40 nm)-conjugated antibody and go to **step 10.**
7. Wash with PBS 4X 5 min.
8. Preincubate with 2% BSA in PBS for 10 min.
9. Incubate with gold (1–10 nm)-conjugated antibody diluted in 1% BSA in PBS for 2 h.

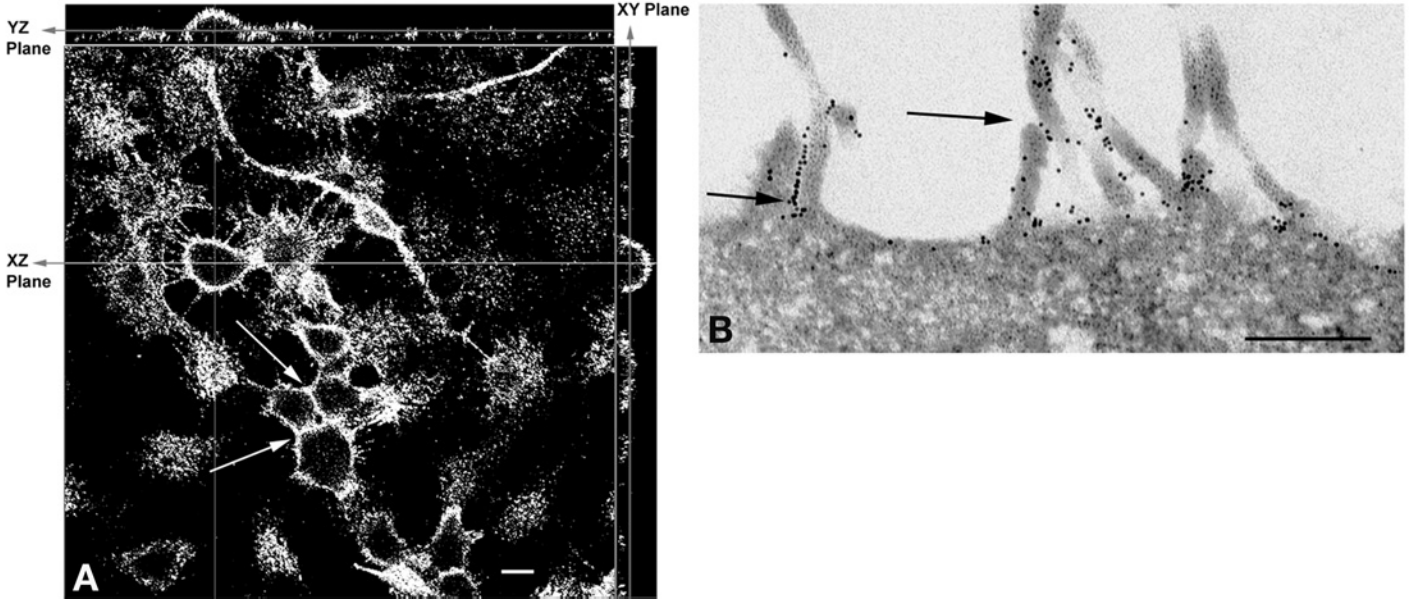

Fig. 12. Reflection contrast image of a surface labeling with immunogold-silver showing the small attachments and detecting small amounts of an antibody on fine structures. These structures as focal adhesions are visible when using 10 nm gold with silver enhancement. Scanning was performed with the 488-nm laser line in the reflectance mode and a pinhole size of 10 µm. U2 cells after mild fixation and labeled with anti-Ep-CAM, and gold (10 nm)-conjugated antibody and a short silver enhancement. **(A)** Typical CLSM image showing an ortho display of a z-scan. The line in the *XZ* and *YZ* plane show the *Z*-height of the *XY* image (large image) (bar = 10 µm). **(B)** High-magnification TEM image of cell membrane staining without silver enhancement after embedding in Epon (*see* **Note 27**) (bar = 0.2 µm).

10. Wash with PBS 4X 5 min.
11. Fix with 2% glutaraldehyde in PBS for 10 min.
12. Wash with PBS for 2X 5 min and in distilled water 4X 5 min.
13. Enhance with silver in the dark for 20–40 min, as described in **Subheading 3.1.6.4.**
14. Counterstain with 1 µg/mL propidium iodide (fluorescent stain).

Mounting single cells or very small objects in immersion oil (*see* **Note 24**):

15. Wash with distilled water.
16. Dehydrate through 40%, 75%, 95%, and 2X 100% ethanol for 2 min (each step) (*see* **Note 28**).
17. Incubate in a mix of 1:1 ethanol/immersion oil (refractive index = 1.518) for 10 min.
18. Incubate in a mix of 1:2 ethanol/immersion oil for 10 min.
19. Incubate in pure immersion oil for 10 min.
20. Take the cover glasses carefully out of the Petri dish/wells (using a fine forceps).
21. Maintain immersion oil on cells.
22. Put a small drop of immersion oil on the object slide.
23. Place the cover glass (cell side up) on the drop of immersion oil and seal with a small amount of nail polish.
24. Store the slides horizontally in a dry box so that cells are maintained in and covered with oil.
25. Scan the slides with a CLSM in the reflection mode, in combination with differential interference contrast or with fluorescence using the multitrack method. Be careful with the objectives, because cells can be damaged easily, or cover with cover glass and seal with nail polish.

3.1.7.2. Internalization of Endocytic Tracers (Gold)

1. Culture cells on 10-mm-diameter cover glass in a Petri dish.
2. Cells were pulsed in a medium containing BSA/5 nm gold or a gold-conjugated antibody for 1 h at 37°C (*see* **Note 29**).
3. Wash with medium and culture for 16 h at 37°C.
4. Stop endocytosis by washing with cold Hank's medium and fix with 1% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, for 10 min.
5. Wash with Hank's medium for 1X 2 min and in distilled water for 4X 5 min.
6. Continue from **Subheading 3.1.7.1., step 13** and/or for other possibilities (*see* **Note 27**).

3.1.7.3. Internalization of Peroxidase-Conjugated Antibody

1. Culture cells in a 96-well plate.
2. Place the plate on ice, add peroxidase-conjugated antibody (type of antibody that binds to the cell and will internalize) to the medium, and incubate for 1 h.
3. Wash with fresh medium and place the plate at 37°C.

4. Wash with cold Hank's medium after different time intervals (1, 4, and 24 h) for 2X 2 min.
5. Fix after each time interval with 1% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, for 10 min at 4°C.
6. Wash with 0.1 *M* cacodylate buffer (pH 7.4) 2X 10 min at room temperature.
7. Develop with freshly prepared DAB (add 10 µL H_2O_2 to 100 mL DAB solution) for 30 min in the dark and wash with PBS.
8. Postfix with1% osmium tetroxide in 0.1 *M* cacodylate buffer, pH 7.4, for 10 min.
9. Wash 4X 5 min with distilled water.
10. Dehydrate with ethanol from 50%, 70%, 80%, to 95% (each step for 5 min) and 100% ethanol for 2X 10 min.
11. Infiltrate with 1:1 ethanol/EPON for 2 h or overnight.
12. Fill the wells with pure EPON and polymerize at 60°C for 24–48 h.
13. Use a minisawing device to remove the wells from the plate and remove the remaining plastic from the embedded EPON blocks. Proceed with sectioning (**Subheading 3.1.5.1., step 4**) (*see* **Note 16**).

3.2. Observation of Living, Unstained Cells

Living cells can be studied by RCM through the cover glass on which they are growing. A cover glass is thin enough to obtain a focused image of the cells. The underside of the cells is studied (e.g., processes of adhesion, migration, and locomotion). The contact sites of the cell with the glass are dark in RCM, whereas the aqueous surrounding is bright. ***(8,14)***. Special chambers were designed and an inverted RCM was used to visualize living cells ***(7)***.

1. Grow the cells on round cover glasses (25 mm in diameter) in a Petri dish.
2. Heat the stage of the microscope (e.g., with temp control 37- 2 [Zeiss, cat. no. 1052-320]) to 37°C. Check the temperature with a small thermometer in a Petri dish with water on the stage.
3. Place the cover glasses into the holders or on object glass with cavity, with the cells facing down (*see* **Fig. 5A or B**) and add some medium from the Petri dish, avoiding inclusion of air bubbles. They will influence the quality of the images and will damage the cells. First, dry off the outside of the cover glasses with tissue, and in case of glass slides with a cavity, encircle the edge of the cover glass with nail polish, to avoid drifting of the glass and drying of the cells.
4. Place the holder onto the microscope stage and put a drop of oil on the cover glass.
5. Gently approach the cover glass with the objective, focus (if necessary, use phase-contrast or differential interference contrast microscopy to locate the cells), and collect images.

3.3. How to Adapt a Fluorescence Microscope for RCM

A microscope stand with incident illumination either upright or inverted, equipped with a strong light source like a mercury high-pressure or xenon

lamp (*see* **Note 30**) is the basis for RCM. The three extra parts needed for RCM are an oil-immersion objective with a quarter-lambda plate in the front lens, a polarization block, and a central stop system (diaphragm module) or cap/prism.

Only Leica has a complete adaptation set for RCM available (Leica reflection contrast device RC; Wetzlar, Germany) for a DAS Mikroskop Type R (DMR) microscope. An antiflex objective (also with a rotateable quarter-lambda plate on the front lens) and polarization block is available from Zeiss. The adaptation of a Zeiss Axioskop provides a good RCM also. However, in principle, all incident light microscopes can be adapted for RCM; a polarization block is available from all microscope firms (sometimes named immunogold-block or epi-polarization block), three objectives (50×, 63×, and 100×) exist, a central stop system can be developed, or a cap/prism can be used instead.

3.3.1. RCM on a Leica DMR

1. Position a protection filter for the polarizer in front of the lamp (the polarizer can be damaged by long exposure to intensive light); remove the lamp housing (Leica 105z or 106z) and slot the protection filter into the light exit window of the lamp housing from the front.
2. Position the RCM module into the space reserved for it, in the horizontal light path between lamp and illuminator.
3. Exchange a filter block in the epi-fluorescence illuminator for a polarization block.
4. Rotate the RC objective into the light path.

3.3.2. Zeiss Axioskop

An alternative for the module is the use of a cap (glass lens) or prism, linked by oil immersion to the bottom of the glass slide, which improves the image contrast in a manner similar to the central stop (*see* **Fig. 13**) (*see* **Subheading 3.3.1., steps 1, 3,** and **4**).

Link the cap or prism with a very small droplet of immersion oil to the underside of the glass slide at the marked circle. Next, place the slide on the microscope stage (*see* **Note 31**).

3.4. Microscopy

3.4.1. Operating the RCM

Optimize microscope settings

1. Center the incident illumination carefully by using the adjusting screws on the lamp housing (*see* **Note 32**).
2. Center the field diaphragm.

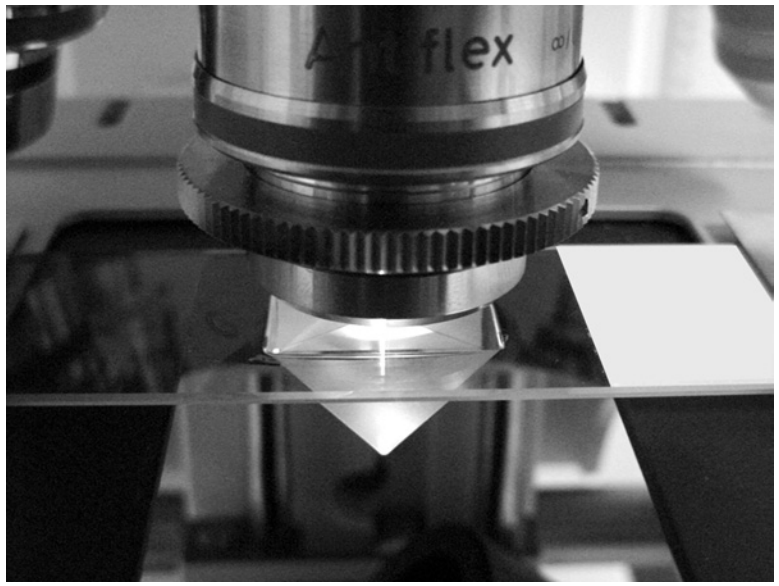

Fig. 13. If no central stop system is available, the use of a prism/cap makes RCM of ultrathin sections possible. The prism is mounted with oil to the glass slide. It functions by directing the light outside of the objective; no reflected light at another place than at the specimen can enter the objective.

3. Choose the size of central stop in the RCM module in accordance with the RCM objective and center its projected image using the Bertrand lens of the microscope in the middle of the image of the arc of the lamp. Find and focus the ultrathin specimen on slide.
4. Circle the sections under a stereomicroscope on the back of the slide with a pen, closely around the specimen, or with a diamond writer if you use a cap/prism. Alternatively, if no stereomicroscope is available, unstained ultrathin sections are detectable by reflecting bright light (e.g., of a lamp) at the section upon tilting the glass slide.
5. Put a drop of immersion oil on top of the sections (the marked area) of the slide.
6. Use bright-field microscopy to find and center the circle with oil (thin sections) with a 5× or 10× dry objective (avoid getting immersion oil on the specimen).
7. Swing in the RCM oil-immersion objective into the observing position and close the field diaphragm slightly and focus. The field diaphragm is simultaneously visible in the field of view. Use the fine focus control and carefully focus. If no image of the field diaphragm is observed, lower the microscope stage (but without losing contact with the immersion oil) until an image of the field stop is obtained.
8. You are now in the plane of the specimen and within the marked area of the slide and should find the specimen by moving the stage slowly in various directions.

Optimize image

9. Rotate the front lens (quarter-wave plate) until the highest reflection is obtained. If the image is not satisfactory (*see* **Note 33**), consider **steps 10** and **11**.
10. For uneven distribution of light, (a) center and align the lamp better, (b) locate the air bubble in the immersion oil between specimen and objective (remove the bubble by swinging objective in and out of the oil), and (c) center the central stop or diaphragm.
11. For poor contrast, (a) rotate the front lens until the highest reflection is observed, (b) change the stop and or aperture diaphragm, (c) improve the contrast by closing down the field diaphragm, (d) control the presence of gray filters in the light path, and (e) improve the lamp alignment by adjustment of the collector of the lamp housing. Finally, collect the images (*see* **Subheading 3.5.**).
12. After examination with the microscope, the slides should be stored vertically in a microscope slide box, to allow the oil to drain away from the slide (except for cells immersed in oil, which should be stored horizontally).
13. For microscopic re-examination of stored dirty slides, first remove the oil with 100% ethanol and allow to dry (*see* **Note 34**).

3.4.2. How to Use the Confocal Laser Scanning Microscope as a Reflection Contrast Microscope

First, find the specimen site for RCM imaging using bright-field microscopy or phase-contrast microscopy. Next, use CLSM in the reflection mode by inserting the NT splitter and removing all dichroic mirrors. To mimic the RCM image with xenon light, choose laser light intensities that combine with white light and reduce the size of pinhole. Refinement of gain(s), amplification offset(s), and focus must be optimized before CLRSM images can be scanned.

1. Put the slide with the circle centered in the beam of the light that is in the center of the condenser front lens. Use bright-field microscopy with low light intensity to find the thin section with a 5× or 10× dry objective. Find and focus the section, or use phase-contrast or differential interference contrast microscopy to find and focus the cells. Use stained (toluidine blue) sections for good visibility; unstained ultrathin sections are visible because of refractions on the edge of sections, in case no circle is set after putting the ultrathin sections on glass (*see also* **Subheading 3.4.1., step 4**).
2. Only lower the stage to change the objective; do not move the stage laterally. Place a drop of oil on the slide and insert the 25× or 63× oil objective and focus again using bright-field microscopy.
3. Locate part of the section of interest in the middle of the field of view and shut the bright-field light down; move the condenser out of position if not needed for differential interference contrast microscopy.
4. Turn on the lasers (argon, He–Ne1, and He–Ne2).

Configuration of the confocal laser scanning microscope:

5. For the LSM-510 (Zeiss), insert the NT 80/20/543 or NT 80/20 neutral beam splitter. For the TCS SP2 (Leica), insert the RT 70/30 neutral beam splitter. For other CLSMs, insert the neutral beam splitter (reflection mode) (*see* **Note 35**).

6. Use one or three detection channel(s) (photomultiplier tubes [PMTs]) (*see* **Note 36**).
7. Code each laser line with its own color in one or each detection channel (488 nm, blue; 543 nm, green; 633 nm, red).
8. Optimize the laser intensity values as when using white visible light. For ultrathin stained sections, use the following illumination intensity values for the LSM510 (Zeiss): 6% of the 488-nm line, 15% of the 543-nm line, 2% of the 633-nm line.
9. Optimize the detector pinhole diameter for the PMT in use. Use 25–100 μm for weakly stained ultrathin sections and use a smaller pinhole (10–30 μm) for gold or gold/silver staining (*see* **Note 37**).

Scan control:

10. Set detector gain to 50%. Use the fast-scan mode and focus with the fine-focus knob untill maximum reflection intensity (focused image) is achieved.
11. Optimize the detector gain of the PMT in use during continuous scanning until there is a bright and contrast-rich image of all known structures that are seen separately (optimized detector gain, "gain").
12. Split into RGB images. Optimize the amplification offset for each color so that the background (reflection of the glass) is black or dark for each laser line (optimized amplification offset, "black level").

Scan image:

13. Make a single image with a low-scan speed and choose the minimal frame size of 1000×1000 and minimal 8-bit pixel depth for high definition.

3.5. RCM Images

3.5.1. Conventional Photographic Recording

1. Focus the reflected graticule (square with double lines in the middle of the overlay) by adjusting the eye lenses of the observation eyepieces until the double lines of the focusing aid are sharply and separately focused.
2. Adjust the image focus with the coarse/fine controls of the microscope.
3. Close the field diaphragm just around the format outline.
4. For exposure time, use spot measuring if there is high contrast in the image (in most cases) or use integral measuring. Point spot on the highest reflection part; only if a part of the spot covers highly reflective gold/silver particles, adjust for the percentage that is covered.
5. Take picture (*see* **Note 38**).
6. Dia positive film can be easily digitized on a high-resolution slide scanner.

3.5.2. Digital Recording

Reflecting contrast microscopy images show a high contrast that should be kept in the recording; therefore, use a high dynamic range. Also, RCM images have very high definition, so that a high-resolution camera is needed (charge-coupled devise basic resolution of 1.3 megapixels [8-bit color]). The purpose of

the recording is to obtain an image as good as the one observed by eye through the oculars, with the same colors and dynamic range.

1. Start with a desired image in focus in the eyepieces.
2. Insert the camera in the light path (parfocal) and direct all of the light to the camera.
3. Operate the camera according to the manual; set automatic white balance.
4. Fine-focus the image on the screen during real-time imaging.
5. Set the black level, gamma for brightness according to the real observed image.
6. Capture the image.
7. For a bright-field-microscopy-like image, invert the image. For an EM-like image, invert the image and convert to black and white (*see* **Fig. 1A,C**).
8. Because of high contrast, image analysis of RCM images is easily performed; there are fewer steps for image improvement. Begin, perhaps, with segmentation.

3.5.3. Image Interpretation

The color of the tissue is complementary to the bright-field color because the wavelengths that are strongly absorbed are also strongly reflected. The effect of osmium tetroxide fixation on the RCM image is an enhancement of contrast of membranes and the peroxidase–DAB product, resembling more that of an inverted TEM image.

Interpretation of immunocytochemical staining in a RCM image is best done by comparing with a RCM image of a control section; namely all incubation steps performed but without the specific primary antibody.

In this chapter, we did not show colored images; however, the colors in the RCM-image facilitate interpretation. For example; the gold color can be easily detected in an immunogold (15–40 nm)-stained section. Undecalcified bone, in an ultrathin Epon section counterstained with toluidine blue, is light blue and cells are orange. These examples in black and white do not have a high-contrast difference but a good color distinction.

4. Notes

1. To study ultrastructure, EM is the best way, because RCM is a light microscopic method and the resolution depends on the wavelength of the imaging rays/particles and on the numerical aperture (NA). The NA of the RC objective should be 1.4 for highest resolution. However, CLSM is even better with the same objective, because as a result of the scanning principle, the resolution is $\sqrt{2}$ times higher.
2. Reflection contrast microscopic images generally show the background of the tissue as black and the tissue itself as white, resembling a fluorescent image or an inverted TEM image. If the background of the image is bright and the object dark, it looks like a bright-field image; then the reflection at the interface of the glass slide is the strongest. With gelatin-coated glass slides, this effect is obtained.
3. A cap can be used instead of a central stop system. It can be a prism or a small lens, and its function is to prevent light that reflects beneath the object from entering the

objective. The main interface that causes these reflections is the one between the bottom of the glass slide and air (*see also* **Fig. 13** and **Subheading 3.3.2.**).

4. For visualization of morphology at the highest definition, the specimen must be ultrathin and the refractive index as close to glass as possible. Between 50 and 100 nm, the strongest, brightest reflection is observed, namely white-light yellowish. This color is the same as the thin section has when it is floating on the water bath of the ultramicrotome. Submerging an ultrathin plastic section or immersing an ultrathin cryosection (sectioned after freezing the tissue) in oil (*see* **Subheading 3.1.7.1., steps 15–23**) or an embedding medium with the same refractive index as glass provides good RCM images of all material present for morphology. Enhancement of contrast of these kinds of image can be obtained by overall staining (e.g., toluidine blue) or specific enhancement of certain structures (e.g., DNA with acridine orange). However, even thicker sections (0.25 μm) cut on a motorized routine microtome provide reasonable RCM images.
5. Mild and reversible fixatives (formaldehyde, acetone, methanol, etc.) as used for paraffin embedding destroy ultrastructure normally not visible with bright-field microscopy, but visible with RCM ***(15)***.
6. All solutions are made with Milli–Q water (i.e., double-distilled filtered water) Blocking buffers and solutions for silver enhancement (*see* **Subheading 3.1.6.4.**) are filtered, except the solutions containing gold particles or antibodies.
7. Fixation can vary and strongly depends on the tissues or cells under study. However, the general rule is to use freshly made paraformaldehyde in the range of 1–4% in 0.1 *M* phosphate or cacodylate buffer, adjusted to pH 7.4. Glutaraldehyde is subsequently added to the proper concentration (0.05–0.2%) for further improvement of the ultrastructural morphology without loss of antigenicity.
   a. *Fixing of tissues*: if perfusion is not possible, then immersion fixation is an option. However, it is important to transfer the sample to the fixative as rapidly as possible after removal and to avoid all forms of mechanical trauma. If necessary, the tissue can be carefully sliced into small slabs as soon as it has been immersed in fixative.
   b. *Fixing of cells*: for preservation of morphology, 10-min fixation is sufficient, and a lower concentration of 0.1–1% glutaraldehyde in buffer can be used. For immunocytochemistry, prolonged fixation in fixative containing glutaraldehyde is not recommended. For cryosectioning, longer than 2 h in fixative containing glutaraldehyde causes the samples to become brittle and difficult to cut. After incubation in fixative, store in store buffer (*see* **Subheading 2.1.1.2.**).
8. Pre-embedding immunogold or peroxidase–DAB labeling is an approach that applies to the localization of both intracellular and extracellular antigens. It also can be applied for the localization of extracellular antigens in unfixed material. Using in vivo, bound, peroxidase-conjugated antibodies, fixation with glutaraldehyde can be performed after the immuno-incubations.
9. The blocking of free aldehydes can be achieved in various ways. PBS–50 m*M* NH_4Cl seems to be the most efficient method. This step reduces immunocytochemical background.

10. When antibody is bound in vivo, the use of detergents is not always necessary, especially when a small amount immunogold (1–5 nm) is used as a label. With a mild fixation, such as 2% paraformaldehyde, the penetration of the Fab fragments of an antibody is 5–7 µm. This is enough to cut several ultrathin sections parallel to the vibratome section.
11. Azide is used to stop bacterial growth and to prevent internalization of antibodies by unfixed cells.
12. When performing pre-embedding labeling, the time involved with incubation and washing might have to be prolonged to warrant complete penetration of reagents to internal antigens and removal of unreacted reagents!
13. Silver enhancement of gold can be applied either before or after osmium tetroxide fixation. Wash extensively after osmium tetroxide fixation because traces of this strong oxidizing agent will interfere with adequate silver enhancement. Osmium tetraoxide could remove the metallic silver deposits; therefore, apply it briefly (20 min) and at a lower concentration (0.5%) at 4°C.
14. For observation with RCM, osmium tetroxide fixation is not necessary. A light counterstaining of the ultrathin sections with toluidine blue or hematoxylin provides RCM images with high contrast and much morphological detail.
15. For example, steps done before starting this protocol: For morphology, fix cells with 1% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, for 10 min; for immunocytochemistry, see **Subheading 3.1.7.1., steps 1–13** or **Subheading 3.1.7.2., steps 1–6** (*see also* **Note 13**).
16. Start sectioning directly with ultrathin cutting, parallel to the monolayer.
17. Avoid introducing oxygen from air, as it interferes with polymerization. Mixing the components is simply achieved by gently stirring with a glass Pasteur pipet. Before using, let the mixture cool in the freezer (–20°C). When using Unicryl, there is a ready-to-use mix, and it is not necessary to exclude air from this resin during polymerization.
18. It is important to use indirect UV irradiation on all sides of the capsules. The illumination system is put in a freezer whose walls are covered with aluminum foil, thus permitting the radiation of UV light to all sides of the capsules.
19. For new users, it is best to ask a person who has done this before, because this is a technique that you have to learn and practice for a while.
20. For hydrophilic embedding medium (Lowicryl K4M, Unicryl), use a low water level and a higher cutting speed of 3–5 mm/s. For Spurr's resin and EPON, use a speed of 1 mm/s.
21. Stabilize your hand on the microtome during manipulation of sections.
22. Preparing small blocks of specimen: Ideally, the pieces of tissue or cell pellets should be trimmed to a pyramidal shape so that when mounted onto the specimen stub, the base is wider than the top. If possible, the block face should be flat and resemble a square, rectangle, or trapezoid.
23. Sucrose infiltration: Although an overnight infiltration in 2.3 *M* sucrose is recommended, it is possible to leave tissue pieces and cell pellets in the sucrose for much shorter times (1–2 h). However, uneven infiltration could occur that will make the sample more difficult to section.

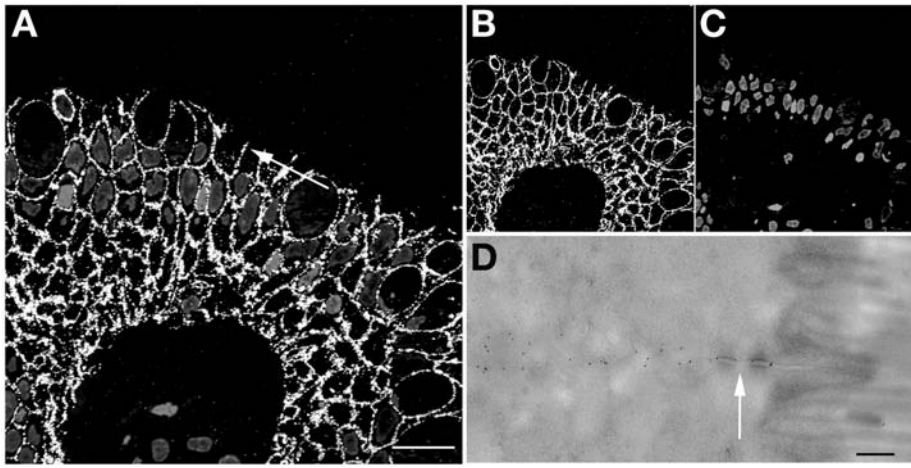

Fig. 14. The fluorochrome propidium iodide shows no reflection at all. Therefore, this fluorescence staining is useful as a counterstain in combination with reflection in the multitracking confocal laser scanning microscope. Lowicryl K4M section (250 nm) of human colonic epithelium stained with MoAb against Ep-CAM conjugated to 10 nm gold and silver-enhanced (post-embedding immunogold–silver method). Propidium iodide was used as a counterstain for DNA, showing nuclei. **(A)** Overlay image of reflection and fluorescence (bar = 20 µm); **(B)** only reflection image; **(C)** only fluorescence image; **(D)** high-magnification TEM image of cell membrane immunogold staining (arrow) without silver enhancement (bar = 0.1 µm).

24. Speel et al. ***(16)*** described other substrates for use with alkaline phosphatase, peroxidase, or glucose oxidase that also show colored reflection in RCM. For most substrates, fixation of the enzyme precipitates in a protein matrix is essential; thereafter, they can be mounted in immersion oil. For mounting sections in immersion oil, use a very small amount of oil, cover with a glass slip, press between filter paper, and seal with nail polish.
25. The peroxidase is still active at this glutaraldehyde concentration, and H_2O_2 will cause less damage because of better fixation.
26. Light green is less reflective and, therefore, a better counterstain with DABox reflection. Toluidine and hematoxylin are strong reflecting counterstains, especially useful with silver-staining. Propidium iodide does not reflect but is suitable in multitrack confocal reflection–fluorescence combination (*see* **Fig. 14**). It is useful for counterstaining nuclei in Lowicryl/Unicryl and cryosections, but it does not stain EPON sections properly. Acridin orange both reflects and fluoresces and provides a strong reflection on thin sections.
27. More possibilities by combining different protocols. For example, after **Subheading 3.1.7.2.**, take cells back to ethanol, wash twice to remove immersion oil, and then embed in Epon **(Subheading 3.1.4.1., step 4)**, without post-osmium tetroxide fixation, until ultrathin sectioning (*see* **Fig. 15**).

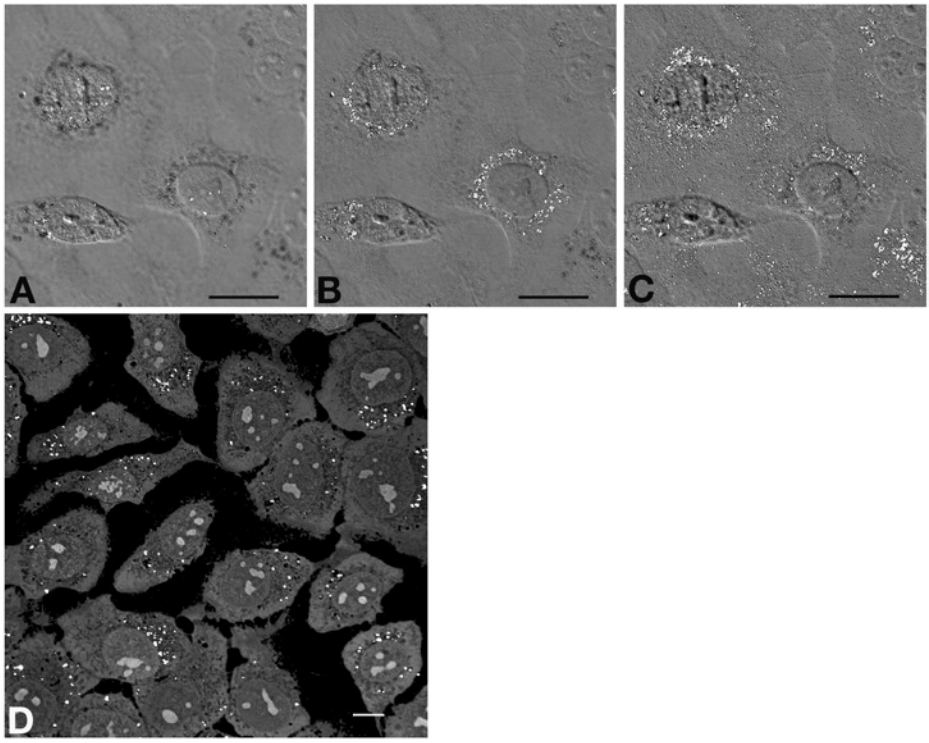

Fig. 15. Example of whole-cell experiment combined with a pre-embedding method. Internalization of BSA–5 nm gold and silver enhancement on whole squamous carcinoma cells (U2). **(A–C)** At three different heights from top to bottom of cells, overlay images of a RCM–confocal slice on differential interference contrast microscopical image (not confocal!). The reflection images are made with a small pinhole (10 µm) (*see* **Note 39**). **(D)** CLS-RCM image of a 90-nm Epon section of the same cells as shown in **(A–C)** after embedding in Epon, and counterstained with 0.01% toluidine blue. CLS-RCM image is made using three laser lines (633 nm, 543 nm, and 488 nm) and detected with pseudo-color gradation RGB in one channel in the reflection mode. (bar = 10 µm)

28. If water is still included in the specimen, then very strong white reflections will occur, which disturb the image. Therefore, dehydrate with 100% ethanol.
29. Small gold particles do not influence the capping process. For observation of strongly stained sections (e.g., toluidine blue-stained ultrathin sections), use a halogen lamp of 3200 K.
30. Mercury high-pressure lamps (150 or 100 W) emit strong emission lines dominating the color of the reflection by the specimen. Xenon lamps (75 W) emit an almost continuous spectrum and provide "true" colors. Upon reflection, the wavelength does not change, so the color of the incident light determines which colors can be seen in the image. The reflectance of the specimen determines what wavelength and in what amount will be reflected.

31. When using this cap/prism method, the specimen must be circled with a diamond writer pen, otherwise the ink will dissolve.
32. Realign the arc of the lamp and the central stop every time the lamp is replaced.
33. How do you know you have a proper/real RCM image? Inverting the image should yield a familiar bright-field microscopic or TEM image. A reference image can be made of a familiar (osmicated) ultrathin TEM section placed on glass and visualized directly with oil on the section.
34. Specimens can be embedded in mounting medium and covered with cover glasses, but they will fade away after months. They can also be embedded in immersion oil, covered with cover glasses, and sealed with nail polish.
35. The NT 80/20/543 has a neutral splitting function. About 20% of the laser energy is reflected toward the sample. From the reflected light, about 80% will pass this same splitter toward the photomultiplier tubes (PMTs).
36. One PMT is used to avoid pixel shift between the different channels.
37. For the gold or gold/silver markers, a use higher laser power and smaller pinhole. This configuration is also optimal for z-scan on "gold-stained" whole cells (*see* **Fig. 12**).
38. To acquire a satisfactory picture, take more pictures with different exposure times. Use the automatic measured exposure time and half and double it.
39. A very small pinhole can be used when there is strong reflection in the specimen. This allows also for z-scanning with more optical slices; however, be aware of the fact that sometimes very strong signals can be detected in more than one optical section.

References

1. Yamaguchi, H., Maat-Schieman, M. L. C., van Duinen, S. G., et al. (2000) Amyloid β protein (Aβ) starts to deposit as plasma membrane-bound form in diffuse plaques of brains from hereditary cerebral hemorrhage with amyloidosis-dutch type, alzheimer disease, and nondemented age subjects. *J. Neuropath. Exp. Neurol.* **59**, 723–732.
2. Natté, R., Yamaguchi, H., Maat-Schieman, M. L. C., et al. (1999) Ultrastructural evidence of early non-fibrillar Aβ42 in capillary basement membrane of patients with hereditary cerebral hemorrhage with amyloidosis, dutch type. *Acta Neuropathol*. **98**, 577–582.
3. Ploem, J. S., Cornelese-ten Velde, I., Prins, F. A., Bonnet, J., and de Heer, E. (1997) Reflection contrast microscopy. *Sci. Tech. Inf.* **XI(4)**, 98–113.
4. Nasser Hajibagheri, M. A. (1999) *Electron Microscopy: Methods and Protocols,* Humana, Totowa, NJ.
5. Filler, T. J., Rickert, C. H., Fassnacht, U. K., and Pera, F. (1994) Reflection contrast microscopy within chrome–alum hematoxylin stained thick tissue–sections. *Histochemistry* **101**, 375–378.
6. Filler, T. J. and Peuker, E. T. (2000) Reflection contrast microscopy (RCM): a forgotten technique? *J. Pathol.* **190**, 635–638.
7. Ploem, J. S. (1975) Reflection-contrast microscopy as a tool for investigation of the attachment of living cells to a glass surface, in *Mononuclear Phagocytes in Immunity* (van Furth, R., ed.), Blackwell, Oxford, pp. 405–421.

8. Verscheuren, H. (1985) Interference reflection microscopy in cell biology: methodology and applications. *J. Cell Sci.* **75**, 279–301.
9. Piller, H. (1959) Enhancement of contrast in reflected-light microscopy. *Zeiss-Werkschrift* **34**, 87–90.
10. Stach, E. (1949) Verkommungen de Kohlen-Auflichtmikroskopie. *Glückauf* **85**, 117–122.
11. Cornelese-ten Velde, I., Bonnet, J., Tanke, H. J., and Ploem, J. S. (1988) Reflection contrast microscopy. Visualization of (peroxidase-generated) diaminobenzidine polymer products and its underlying optical phenomena. *Histochemistry* **89**, 141–150.
12. Carlemalm, E., Villiger, W., Hobot, J. A., Acetarin, J. D., and Kellenberger, E. (1985) Low temperature embedding with Lowicryl resins: two new formulations and some applications. *J. Microsc.* **140**, 55–72.
13. Danscher, G. (1981) Localization of gold in biological tissue. A photochemical method for light and electronmicroscopy. *Histochemistry* **71**, 81–88.
14. Pluta, M. (1989) *Advanced Light Microscopy. Specialized Methods.* Elsevier PWN–Polish Scientific Publishers, Warszawa, pp. 197–210.
15. Hoetelmans, R. W., Prins, F. A., Cornelese-ten Velde, I., van der Meer, I., van de Velde, C. J., and van Dierendonck, J. H. (2001) Effects of acetone, methanol, or paraformaldehyde on cellular structure, visualized by reflection contrast microscopy and transmission and scanning electron microscopy. *Appl. Immunohistochem. Mol. Morphol.* **9**, 346–351.
16. Speel, E. J. M., Kramps, M., Bonnet, J., Ramaekers, F. C. S., and Hopman, A. H. (1993) Multicolour preparations for in situ hybridization using precipitating enzyme cytochemistry in combination with reflection contrast microscopy. *Histochemistry* **100**, 357–366.
17. Prins, F. A., Bruijn, J. A., and de Heer, E. (1996) Applications in renal immunopathology of reflection contrast microscopy, a novel superior light microscopical technique. *Kidney Int*. **49**, 261–266.
18. Kootstra, C. J., Bergijk, E. C., Veninga, A., et al. (1995) Qualitative alterations in laminin expression in experimental lupus nephritis. *Am. J. Pathol*. **147**, 476–488.
19. Bergijk, E. C., Munaut, C., Baelde, J. J., et al. (1992) A histologic study of the extracellular matrix during the development of glomerulosclerosis in murine chronic graft-versus host disease. *Am. J. Pathol*. **140**, 1147–1156.
20. Leer van, E. H. G., Ronco, P., Verroust, P., van der Wal, A. M., Hoedemaeker, P. J., and de Heer, E. (1993) Epitope specificity of anti-gp330 autoantibodies determines the development of proteinuria in active Heymann nephritis. *Am. J. Pathol*. **141**, 821–829.
21. Cornelese-ten Velde, I. and Prins, F.A. (1990) New sensitive light microscopical detection of colloidal gold on ultrathin sections by RCM. Combination of reflection contrast and electron microscopy in post-embedding Immunogold. *Histochemistry* **94**, 61–71.
22. de Jong, D., Prins, F. A., Mason, D. Y., Reed, J. C., van Ommen, G. B., and Kluin, P. M. (1994) Subcellular localization of bcl-2 protein in malignant and normal lymphoid cells. *Cancer Res.* **54**, 256–260.

23. Balzar, M. and Prins F. A. (1999) The structural analysis of adhesions mediated by Ep-CAM. *Exp. Cell Res.* **246**, 108–121.
24. Curtis, A. S. G. (1964) The mechanism of adhesion of cells to glass. A study by interference reflection microscopy. *J. Cell. Biol*. **20**, 199–215.
25. Kruidering, M., Maasdam, D. H., Prins F. A., de Heer, E., Mulder, G. J., and Nagelkerke, J. F. (1994) Evaluation of nephrotoxicity in vitro using a suspension of highly purified porcine proximal tubular cells and characterization of the cells in primary culture. *Exp. Nephrol.* **2**, 334–344.
26. Neelissen, J. A. M., Arth, C., Wolff, M., Schrijvers, A. H. G. J., Jungingen, H. E., and Bode, H. E. (2000) Visualization of percutaneous ^{3}H-estradiol and ^{3}H-norethindrone acetate transport across human epidermis as a function of time. *Acta Derm. Venereol*. **208**, 36–43.
27. Rickert, C. H., Filler, T. J., and Gullotta, F. (1997) Reflection contrast microscopy on silver-stained amyloid deposits in Alzheimer's disease. *Sci. Tech. Inf.* **XI(5)**, 136–142.
28. Diaz, C. L., Melchers, P. J. J., Hooykaas, P. J. J., Lugtenberg, B. J. J., and Kijne, J. W. (1989) Root lectin as a determinant of host-plant specificity in the Rhizobium–legume symbiosis. *Nature* **338**, 579–581.
29. van der Burg, M. P. M., Guicherit, O. R., Frölich, M., et al. (1994) Assessment of islet isolation efficacy in dogs. *Cell Transplant*. **3**, 91–101.
30. Landegent, J. E., Jansen in de Wal, N., van Ommen, G. B., et al. (1984) Chromosomal localization of a unique gene by non-autoradiographic in situ hybridization. *Nature* **317**, 175–177.
31. Landegent, J. E., Jansen in de Wal, N., Ploem, J. S., and van der Ploeg, M. (1985) Sensitive detection of hybridocytochemical results by means of reflection-contrast microscopy. *J. Histochem. Cytochem.* **33**, 1241–1246.
32. Macville, M. V. E., van Dorp, A. M., Wiesmeijer, K. C., Dirks, R. W., Fransen, A. M., and Raap, A. (1995) Monitoring morphology and signal during non-radioactive in situ hybridization procedures by reflection-contrast microscopy and transmission electron microscopy. *J. Histochem. Cytochem.* **43**, 665–674.
33. Cornelese-ten Velde, I., Bonnet, J., Tanke, H. J., and Ploem, J. S. (1990) Reflection contrast microscopy performed on epi-illumination microscope stands: comparison of reflection contrast- and epi-polarization microscopy. *J. Microsc*. **159**, 1–13.
34. Cornelese-ten Velde, I. and Prins, F. A. (1990) New sensitive light microscopical detection of colloidal gold on ultrathin sections by RCM. Combination of reflection contrast and electron microscopy in post-embedding immunogold histochemistry. *Histochemistry* **94**, 61–71.
35. Cornelese-ten Velde, I., Wiegant, J., Tanke, H. J., and Ploem, J. S. (1989) Improved detection and quantitation of the (immuno) peroxidase product using reflection contrast microscopy. *Histochemistry* **92**, 153–160.
36. Ploem, J. S., Cornelese-ten Velde, I., Prins, F. A., and Bonnet, J. (1995) Reflection-contrast microscopy: an overview. *RMS* **30/3**, 185–192.
37. Ploem, J. S., Prins, F. A., and Cornelese-ten Velde, I. (1998) Reflection-contrast microscopy for high definition images with light microscopy: practical tips using new Leica DM R equipment for RCM. *Sci. Tech. Inf.* **CDR 1**, 47–86.

38. Prins, F. A., van Diemen-Steenvoorde, R., Bonnet, J., and Cornelese-ten Velde, I. (1993) Reflection contrast microscopy of ultrathin sections in immunocytochemical localization studies: a versatile technique bridging electron microscopy with light microscopy. *Histochemistry* **99**, 417–425.
39. Ploem, J. S., Prins, F. A., and Cornelese-ten Velde, I. (1999) Reflection-contrast microscopy, in *Light Microscopy in Biology: A Practical Approach*, 2nd ed., (Lacey, A.J., ed.), Oxford University Press, Oxford, pp. 275–310.
40. Ploem, J. S. (1975) General introduction. *Ann. NY Acad. Sci*. **254**, 40.