Reflection-Contrast Microscopy - Review

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In this article an overview will be given of the applications of Reflection-Contrast Microscopy (RCM). A short explanation of the antiflex and oblique illumination methods, used with RCM is presented. The advantage of using ultrathin sections for light microscopy with RCM is discussed. Further details can be found on the website: www.ploem-reflection-contrast-microscopy.com. A video illustrating RCM is available on YouTube: www.youtube.com/watch?v=sNQGu3nDPX8

Antiflex - A first step to Reflection-Contrast Microscopy

In testing green excitation light to obtain red fluorescence, Ploem made an error. He had omitted a barrier filter in a fluorescence epi-illuminator equipped with a neutral (50%) beam-splitting mirror. A barrier filter was needed to block the green light reflecting from the microscopic specimen towards the eyepieces. When he then examined live tissue culture cells growing on a glass coverslip, stained with a fluorochrome, he observed a blurred green image reflecting from the cells. The image contrast was very unsatisfactory. Later, at the occasion of Ploem's visit to Carl Zeiss (Oberkochen), he discussed this accidental observation of live cells with Horst Piller (Zeiss scientist). Piller thought that the bad image contrast observed by Ploem could be caused by unwanted incident light reflected by the glass surfaces in the microscope objective. Piller told him that he had developed a solution for this problem by using an antiflex method for eliminating the reflection from the lenses of an microscope objective This enabled him to observe the weak reflections of various dark coal specimens (Piller,

Figure 1. An early Carl Zeiss antiflex x40 objective developed for the study of the reflection of black coal. Note the low Numerical Apperature (NA) of 0.65, sufficient for reflection microscopy of dark coal

1959). He made a gift to Ploem of this very early antiflex objective (Figure 1).

The first use of the antiflex methodology in biological applications

Using this early antiflex objective from Carl Zeiss, Ploem could obtain interference images with high contrast of the adhesion zones of live cells growing on glass. A literature search led to the pioneer research that Curtis had carried out much earlier on

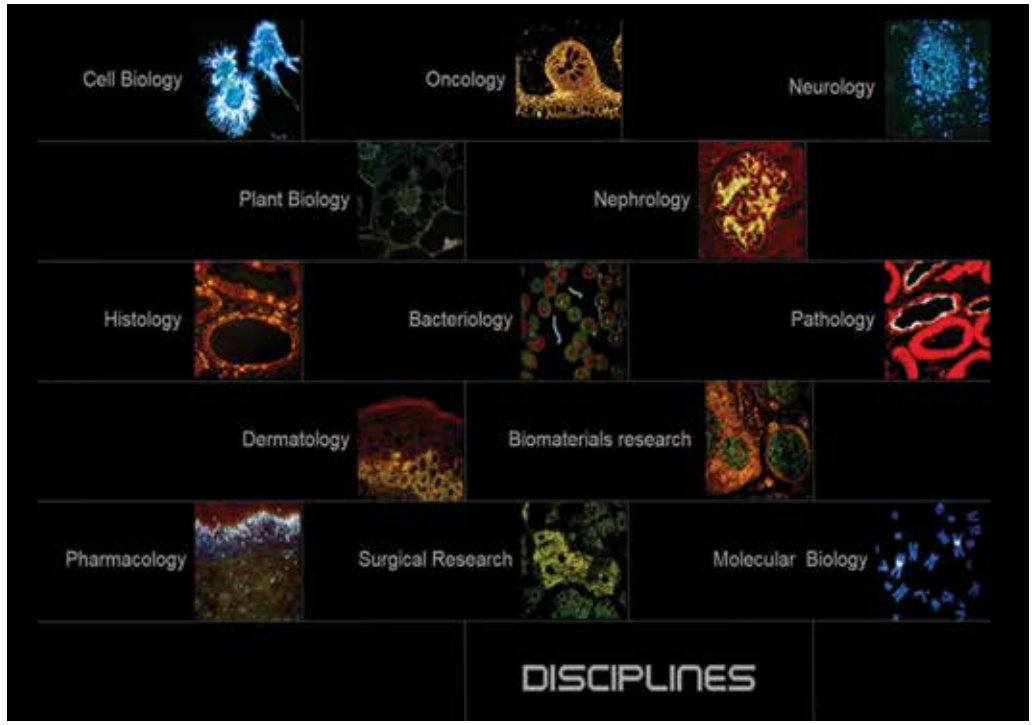

Figure 2. Applications of RCM

the adhesion of living cells to glass surfaces (Curtis, 1964). Curtis described his method as Interference Reflection Microscopy (IRM). Verschueren, 1985, also investigated the contacts of live cells to glass, using conventional epi-illumination. He mentioned that reflections from the lenses of the objective may cause unwanted stray light. He concluded that even relative weak stray light could damage the reflected image, unless the field diaphragm for incident light is narrowed to impracticable low sizes. He mentioned Ploem's introduction into IRM of an antiflex device (Ploem, 1975) as an ingenious, and very satisfactory solution for the stray light problem. Whole cells at high magnification could be visualized (Verschueren, 1985).

Ploem's very first use of the antiflex method in biological and medical studies resulted in defining a microscope method to obtain a significant increase of the image contrast of weak reflections from biological specimens. Such images can result from interference, specular reflections by silver or gold grains, or interference and specular reflections from peroxidase reaction products (Corneleseten Velde, 1990b) [Thesis]. She performed pioneer studies on RCM. She stated that precipitated diaminobenzidine (DABox) reflection by RCM is based on a combination of interference phenomena occurring in the layer of DABox, and selective reflection. The fact that besides interference images, many other interesting types of reflection can be observed, led to this new microscope method being termed in a publication as Reflection-Contrast Microscopy (Ploem, 1975). RCM has now been used In a large number of applications (Figure 2). A Google Scholar search for "reflection contrast microscopy" lists about 750 results (publications), including a limited number of publications describing IRM.

The antiflex microscope method

In a conventional epi-illuminator a neutral (50%) beam-splitting mirror was used for early IRM. This mirror deflects about 50% of the light through the microscope objective towards the microscopic specimen. Some light is reflected from the surfaces

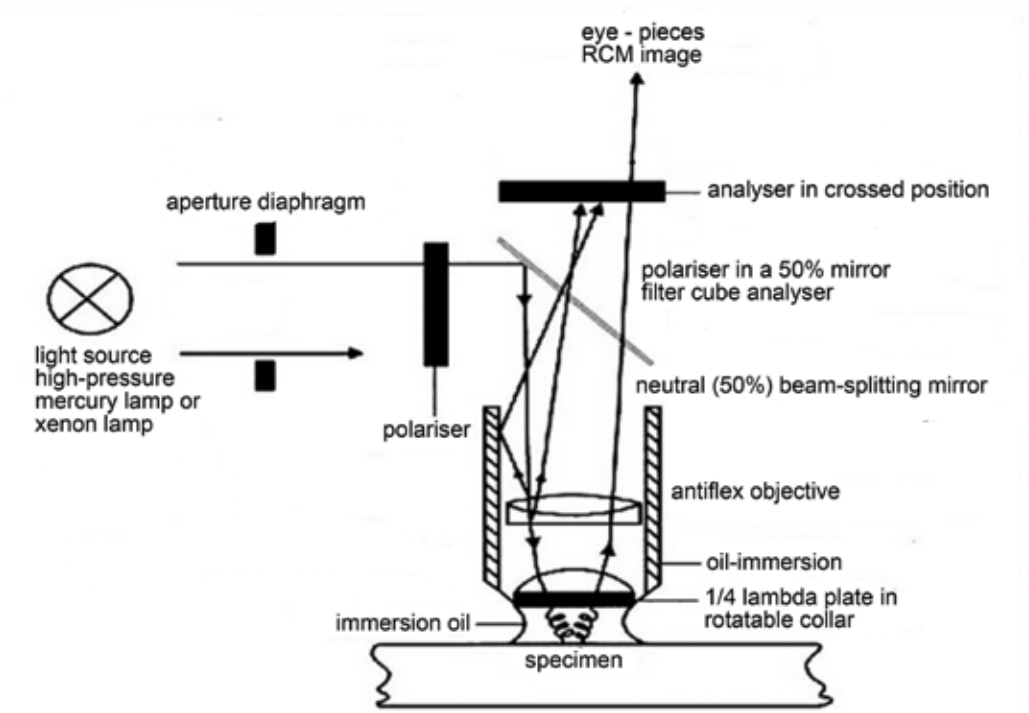

Figure 3. Light path in a reflection contrast microscope with an antiflex filter cube

of the lenses of the microscope objective towards the eyepieces About 50% of the light is not deflected by the neutral (50%) beam-splitter mirror but is transmitted through the beam-splitting mirror onto the inside of the microscope tube. A portion of this light may be reflected upwards and reach the eye-pieces. Both these unwanted types of reflection will diminish the contrast and resolution of the reflected image of the specimen. The antiflex method (Figure 3) uses a filter cube in the epi-illuminator (Figure 4) containing [1] a polariser filter, [2] a neutral (50%) beam-splitting mirror and [3] an analysing polarisation filter. The analyser is mounted in a crossed position and cuts off the unwanted light reflections described above. About 50% of the light is reflected by the neutral (50%) beam-splitter mirror through the objective towards the microscopic specimen. A part of this light is reflected again by the microscope specimen towards the front lens of the antiflex objective. This objective has a rotatable quarter-lambda wave plate mounted on the front lens. The light beam that forms the reflected image from the microscope specimen has to pass through the quarter-lambda wave plate and becomes circularly polarised and can then pass through the polarisation analyser in the filter cube and be observed through the eye-pieces.

Figure 4. Filter cube for RCM, containing a polariser, a neutral (50%) beam-splitter and an analyser. This filter cube can be inserted into one of the positions of a epi-illuminator for fluorescence microscopy and will perform the antiflex functions described in Figure 3

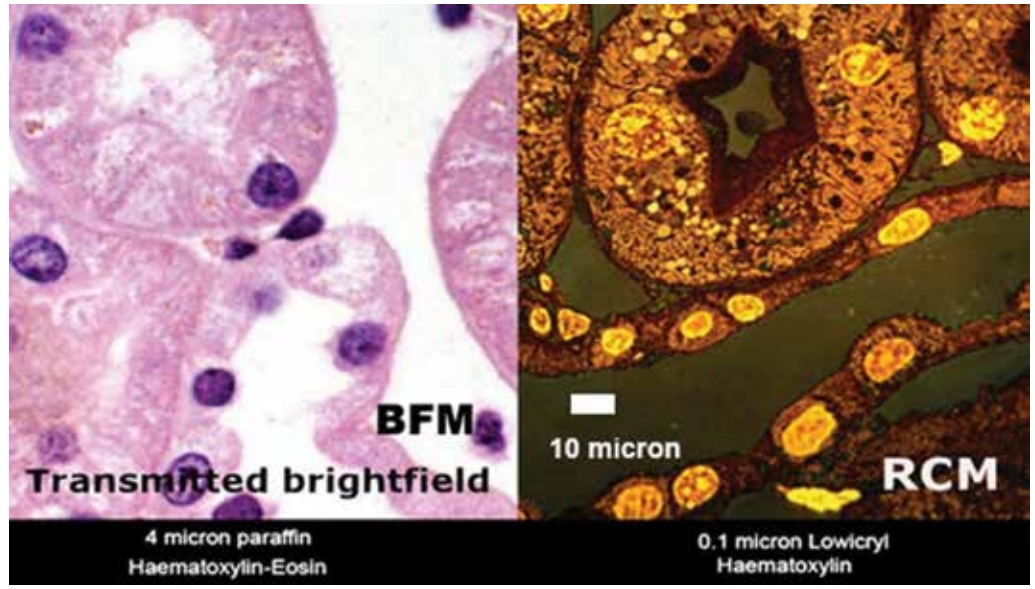

Figure 5. Rat kidney tissue stained with Haematoxylin-Eosin. A 4 micron specimen examined with oil-immersion objective with a depth of field of 0.15-0.25 micron, may show out-of-focus blur (left). Images from ultrathin 0.1 micron thick sections obtained with RCM provide very sharp images and fine morphological details (right) (there is only one focus plane observed)

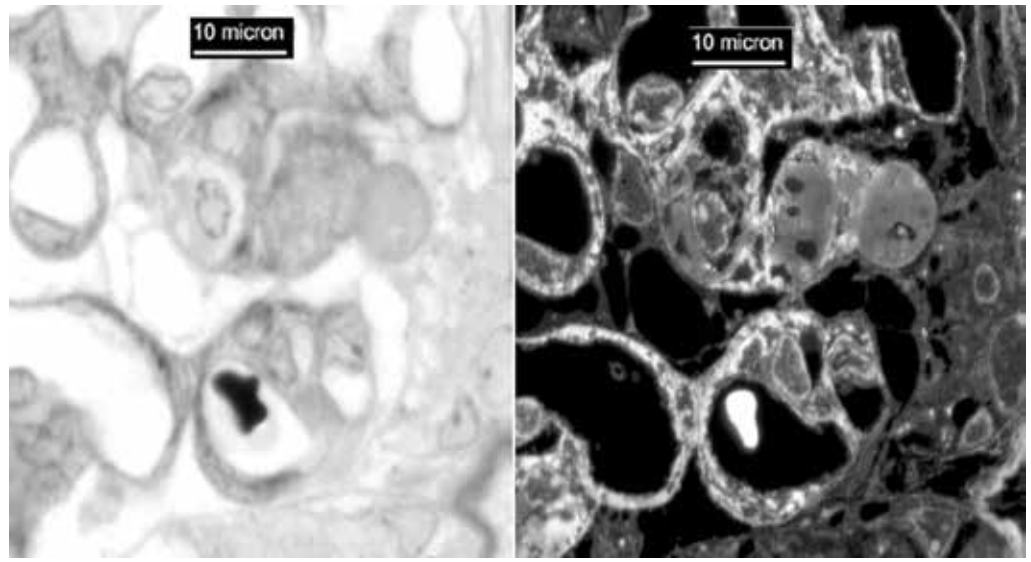

Figure 6. An image obtained with BFM from a specimen with a thickness of 1 micron (left) is still visible but with a low resolution The RCM image of an ultrathin EM section (right) with a thickness of only 0.1 micron shows a good contrast and resolution

Oblique illumination for RCM

To be able to observe very low levels of reflected light, an extremely dark image background is essential. To achieve this, the image contrast should be further increased. A solution can be to use an oblique illumination method (Clarke, 2012 and Patzelt, 1978). The light that is transmitted through the thin ring outside a large round opaque center stop of the aperture diaphragm of the epi-Illuminator, can produce oblique illumination, because of its off-axis position. According to experiments by Clarke, circular oblique lightening, using an opaque round central stop, improves the image contrast and results in a better resolution (Clarke, 2012).

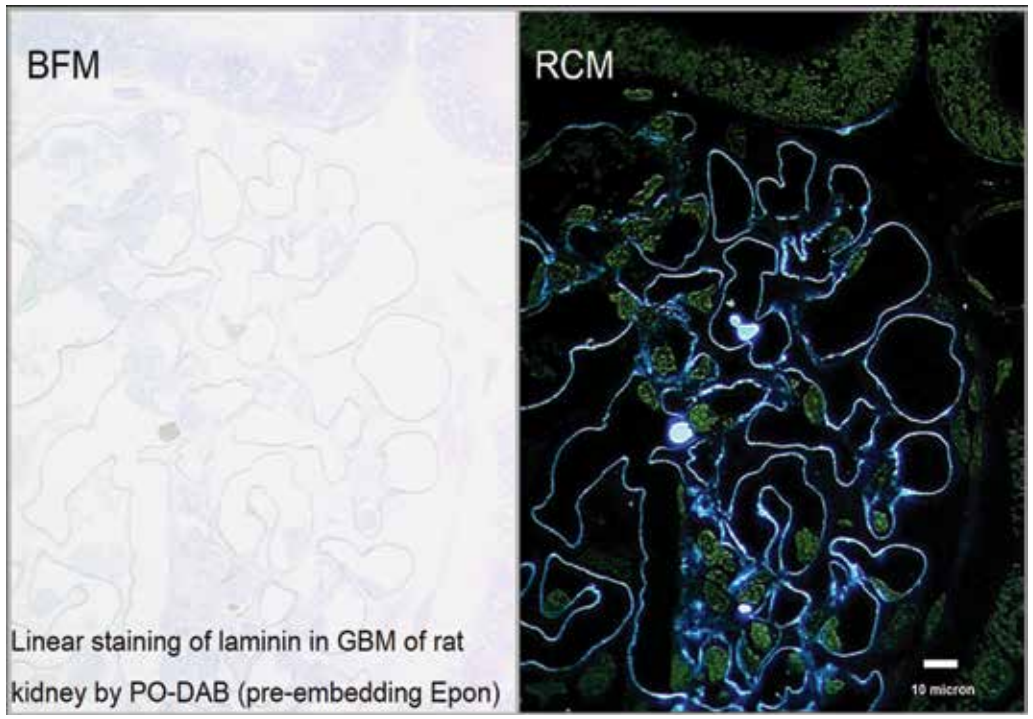

Figure 7. Kidney tissue of a rat. The 0.1 micron ultrathin section is barely visible with BFM (left). The 0.1 micron ultrathin section (right) shows a good contrast and resolution with RCM

Absorption, fluorescence and reflection of cytochemical stainings

To absorb light in a microscopic specimen with Bright Field Microscopy (BFM) a minimal volume of stained cellular material is needed. Routine pathology sections are often 3-5 micron thick. For routine diagnostic use, they provide satisfactory images. To test how much thinner tissue sections can be made and still be visible with BFM, sections were cut 4, 1 and 0.1 micron thick. In a section of 4 micron thick, for routine diagnostic purposes a satisfactory BFM absorption image can be obtained (Figure 5, left). A 1 micron thick section observed with BFM shows a reasonable absorption image (Figure 6, left). A section of 0.1 micron thickness, however, contains very little stained material and shows (with BFM) hardly any absorption (Figure 7, left). The right hand image in these three figures (5, 6 and 7) is obtained with RCM of 0.1 micron thick ultrathin sections, they show a high contrast and a good resolution. Fluorescence Microscopy (FM) is much more sensitive than BFM. According to the Olympus Microscopy Resource Center article "Introduction to fluorescence microscopy" (www.olympusmicro.com/primer/techniques/fluorescence/fluorointrohome.html) a very small number (about 50) of fluorescent molecules per cubic micrometer can be detected in a microscope sample. A smaller number of reflecting molecules can, in many applications, be detected with RCM.

Detection of a single copy gene with RCM

Landegent, et.al., 1985a, succeeded in detecting a single copy gene with RCM. The strong reflection of the Diaminobenzidine (DAB) reaction product after *in-situ* Hybridisation (ISH) could be observed at high contrast (Figure 8). They reported that RCM for the detection of the DAB/peroxidase product also enabled DNA counterstaining and the creation of a chromosomal banding pattern. They mentioned that earlier they could not detect a single copy gene with epi-fluorescence microscopy. A single copy gene has an extremely small mass of DNA. In the literature a mass in trillionths of a gram [10^{-12} gram] is mentioned. In the Life Sciences Guideline Manual from the Roche company this is written about ISH: "The sensitivity demanded for the detection of the mass of a single copy gene requires the detection

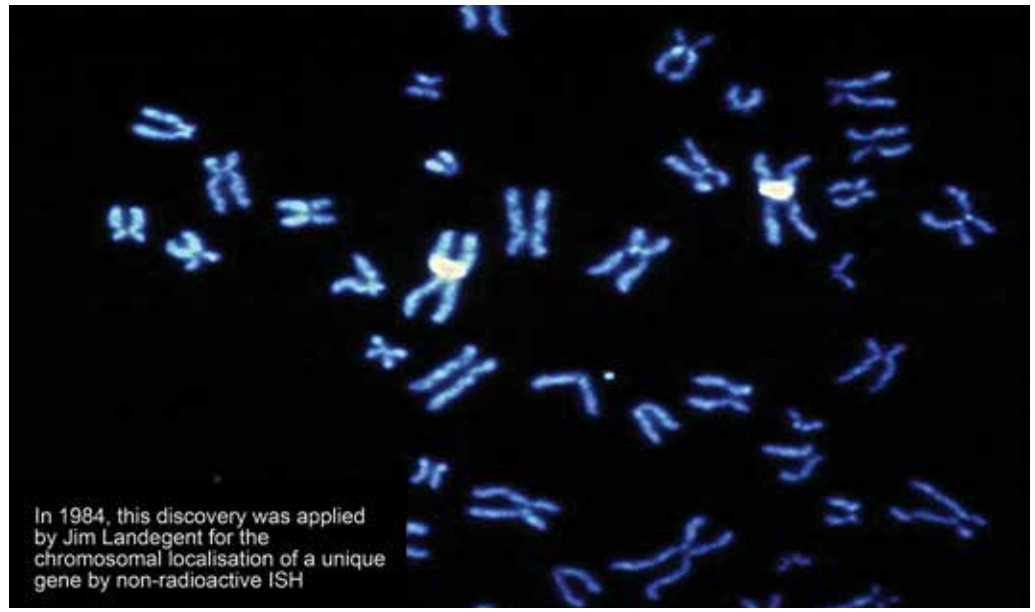

Figure 8. Metaphase chromosomes plate with the detection, using RCM, of the small DAB reaction product (bright spots) of a single copy gene after ISH (Landegent, et.al. 1985a). Note the very dark background obtained with RCM

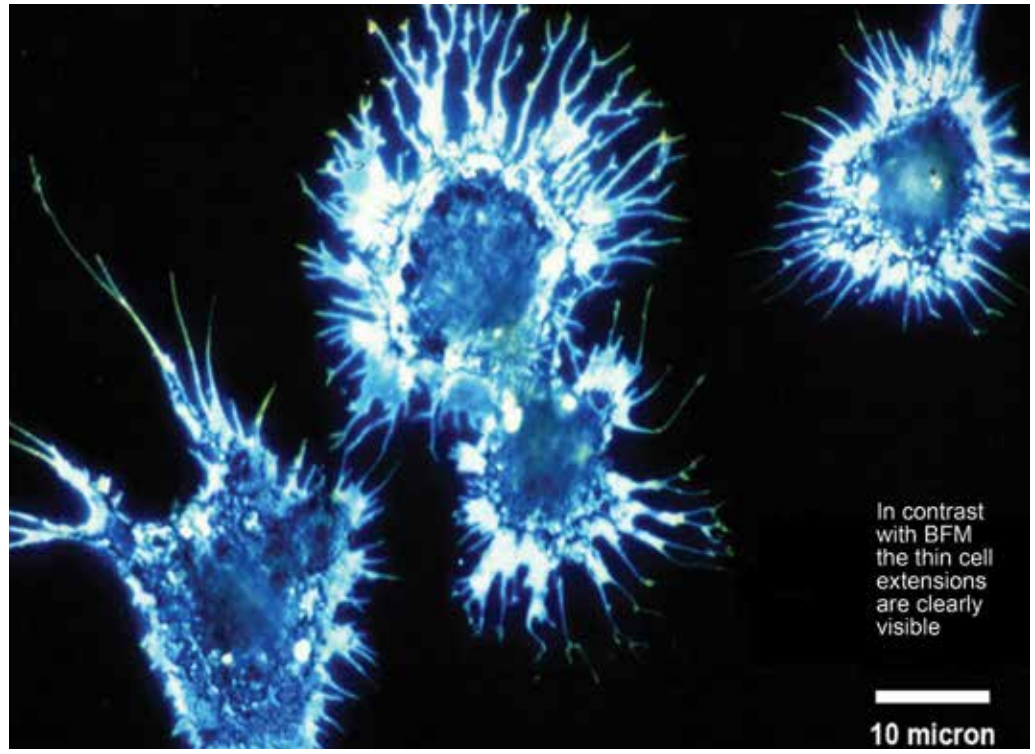

Figure 9. Immunoperoxidase staining of macrophages. It was expected to observe a type of localisation on the cell membrane. Instead, long and fine extensions from the cell surface could be observed. This is the very first microscopic specimen in which the strong reflection of a peroxidase immuno-staining was observed using RCM

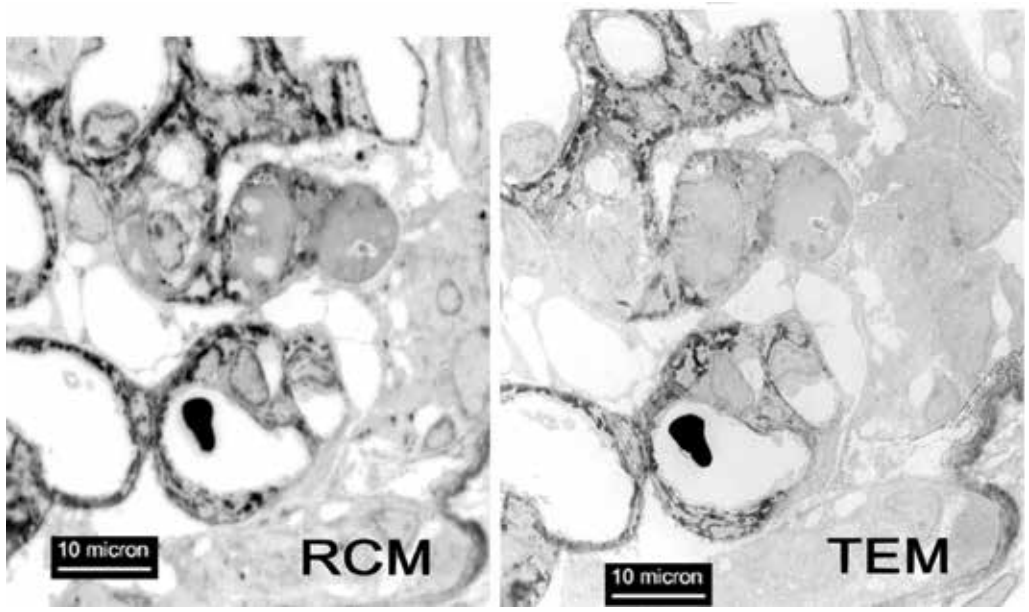

Figure 10. (Left) An image of an ultrathin section (90 nm) obtained with RCM is compared with a low magnification TEM image (right) of a sequential ultrathin section. Quite similar microscope images can be obtained

of 10^{-15} gram of DNA. This may require more sophisticated microscopy. The DAB/peroxidase product displays bright reflection when present in extremely low amounts. This property has made RCM instrumental in detecting the first single copy gene by non-radioactive means"

RCM for sensitive light microscopy

The pioneer investigations of Bauman, Wiegant, et. al. 1987, Raap, et. al. 1989. and Nederlof, et. al. 1990, on ISH about the detection of the cytochemical reaction products with epi-fluorescence microscopy, created a large scientific data base on ISH, which later, using RCM, could be further developed. The first indication of the sensitivity of RCM was shown by an immunoperoxidase staining of macrophages. It was expected that this created a localisation of the staining around the cell membrane. Instead a very bright reflected image was obtained with RCM, far outside of the main cell membrane area (Figure 9). Landegent, et. al. 1985b wrote a publication entitled "Sensitive detection of hybridocytochemical results by means of RCM". They state that it requires only a rather simple micoscope technique. Ambros, et. al., 1987, also used nonisotopic ISH and DAB detection with RCM and succeeded in obtaining a chromosomal insertion of human papillomavirus 18 sequences in HeLa cells. They could, in addition realise subsequent fluorescent R- and C-banding. Cornelese-ten Velde, et. al., 1988, confirmed that RCM is indeed a very sensitive method for detecting extremely small amounts of the DAB oxidation product. Van Dorp et. al., (1996), found that in comparison with BFM and FM, RCM is a more sensitive method.

RCM in combination with Transmitted Electron Microscopy (TEM)

Prins carried out pioneering research using both RCM and TEM on sequential ultrathin sections (Prins, et. al., 1993). He suggested that RCM could serve as a bridge to TEM. A similarity is often found between images obtained with RCM and low magnification TEM (Figure 10). Only slightly adjusted electron microscopy methods for ultrathin specimen preparation are needed for RCM. Macville, et. al., 1995, analysed the effects of ISH with RCM and TEM. Ultrathin sections were examined by RCM followed by the observation of sequential

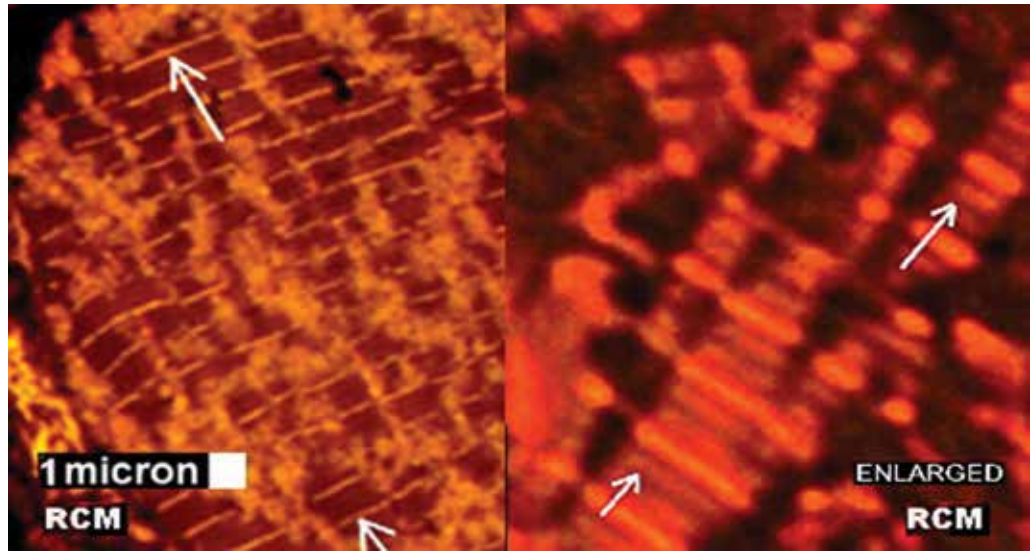

Figure 11. Ultrathin Epon section of a hamster heart, stained with Toluidine Blue. Here the fine structures of Z and H banding in muscles (arrows) can be observed with RCM

ultrathin sections by TEM. Yuruker,et. al., 2008. compared RCM with TEM to understand whether RCM could be used for routine histopathological diagnosis of various kidney diseases, could it be a less expensive and simpler alternative to TEM? Their findings showed that this was quite possible using about 50-100 nm thick ultrathin sections. They state that RCM showed similar results to TEM. Koerten,

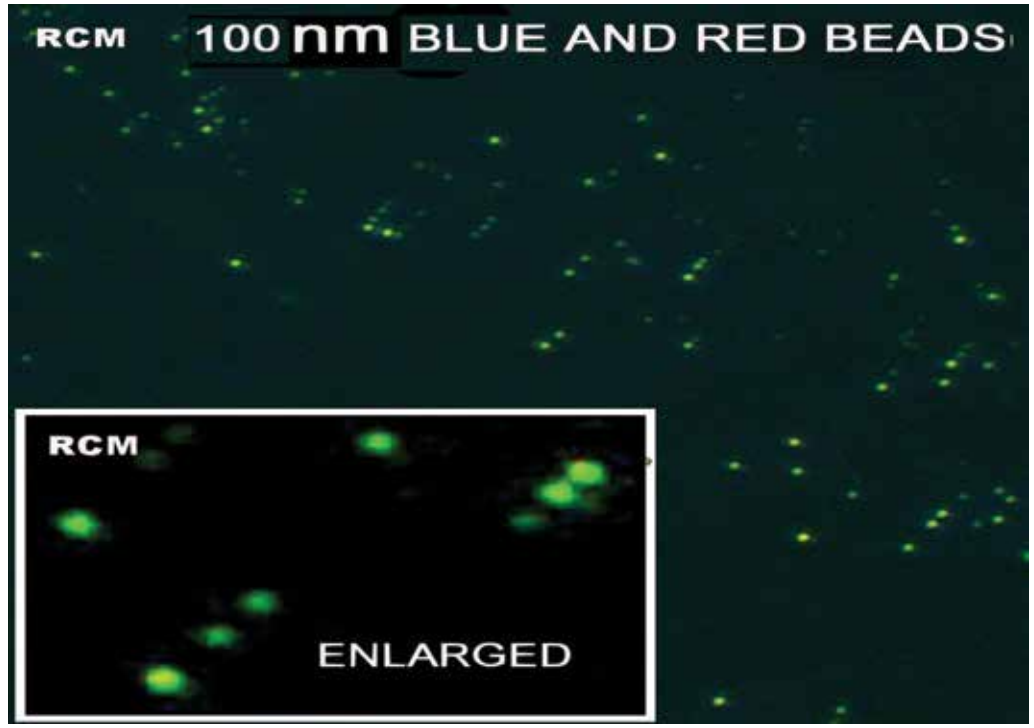

Figure 12. Red and blue stained microbeads (reflecting green light) with a diameter of 100 nm are obtained from Micromod Partikeltechnologie GmbH,D-18119 Rostock, Germany), two 100 nm beads (in the right hand upper corner of the enlarged image) reflecting green light, are very close to each other, but do not overlap. Due the high image contrast with RCM the gap between these two beads is visible. This image suggests that RCM resolution is close to the maximum of that of a conventional light microscope

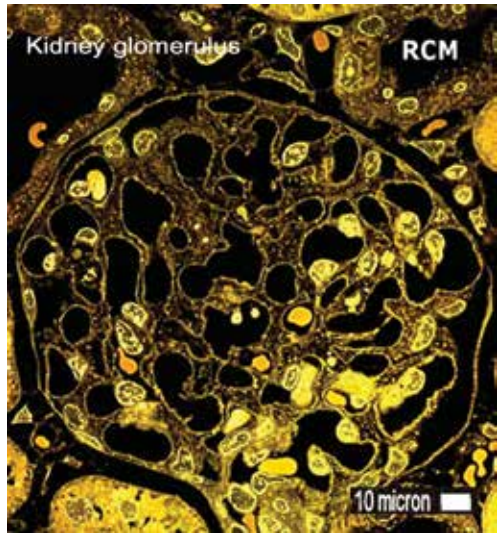

Figure 13. Ultrathin section of 90 nm tickness from a kidney glomerulus. The good image contrast, high resolution and colour presentation is well suited for diagnostic examination. No out-of-focus blur is observed in this section. Toluidin blue staining

et.al. 1980, investigated the ingestion of latex beads by filopodia of mouse peritoneal macrophages. They observed that RCM and TEM showed the same fine filopodia for a given cell.

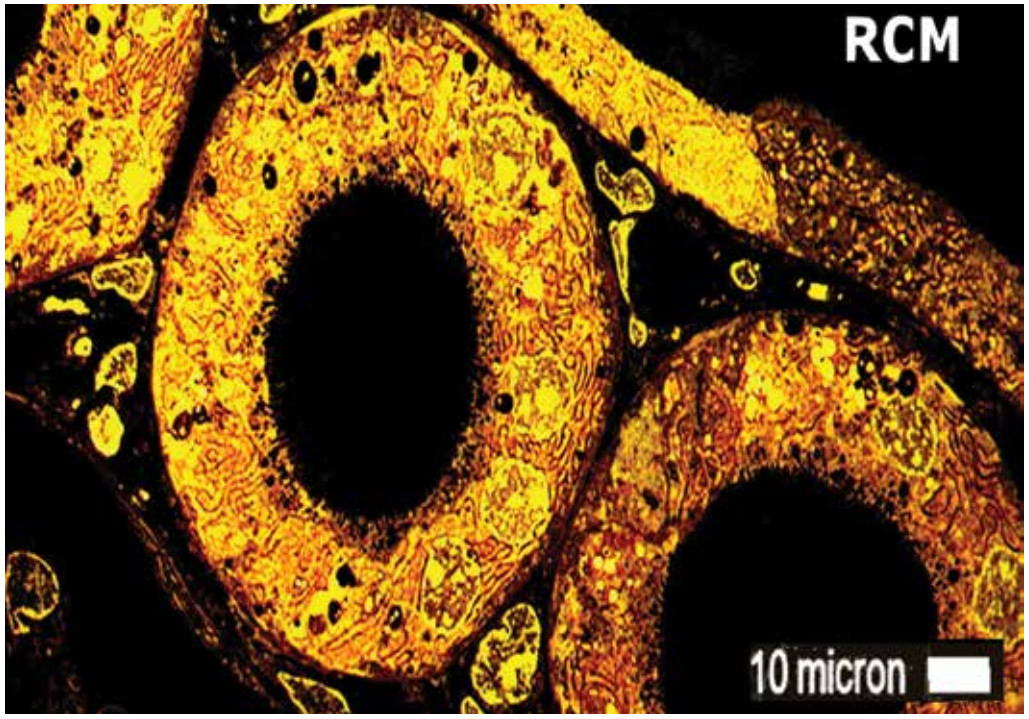

Figure 14. From this ultrathin section (90 nm). No out-of-focus blur is produced, permitting the observation of fine image details and a high image contrast. Toluidin blue staining

Resolution of RCM images

With most light microscope methods, including BFM and FM, a resolution of about 250 nm is considered acceptable for routine use. The best resolution for conventional light microscopy is about 200 nm. For BFM the resolution is dependent upon the total optical train of the microscope, including a well centered sub-stage condenser with a high NA. The optimal resolution of images obtained with RCM depends on using epi-illumination with a high NA antiflex objective. It profits from a high image contrast achieved with RCM and the absence of out-of-focus blur when 0.1 micron thick specimens are inspected. RCM can visualise very fine structures like the Z and H bands in muscles (Figure 11), which are difficult to observe with routine conventional BFM.

The image of a specimen of microbeads with a diameter of only 100 nm, demonstrates that by using RCM a microscope image resolution can be obtained that is close to the maximum resolution of conventional light microscopy (Figure 12).

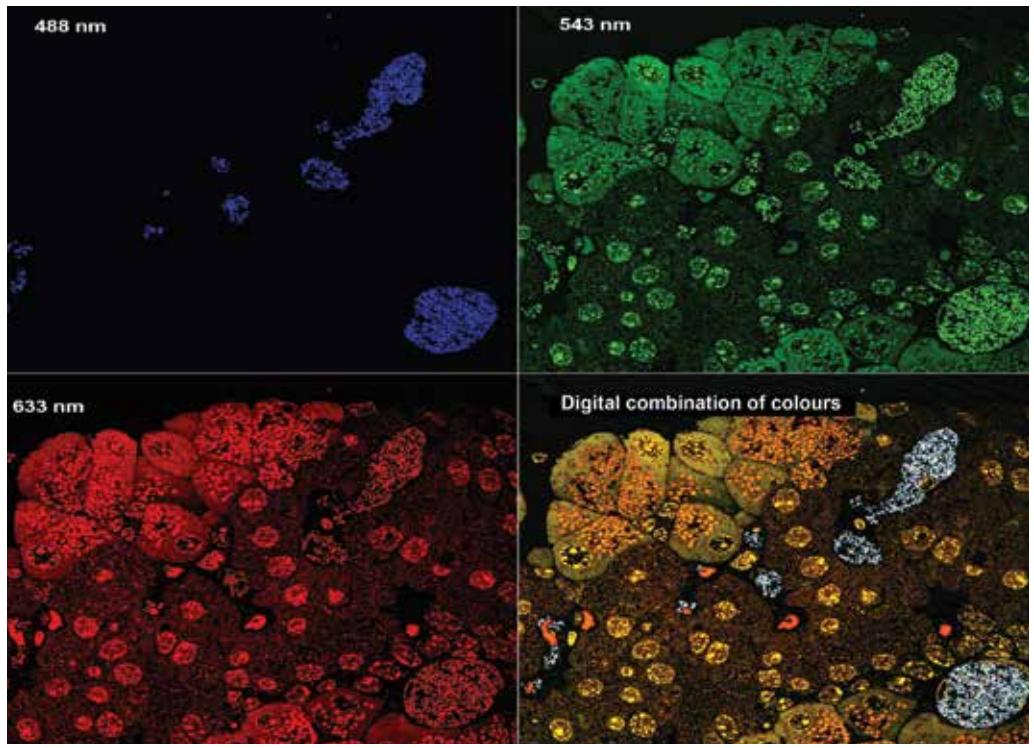

Figure 15. Using three consecutive laser wavelengths (laser lines 633 - 543 - 488 nm) for the confocal microscopy of a section with a DAB reaction product staining. By digitally combining the three images, a multi-colour image is generated (bottom right)

Oil immersion objectives with a 0.15 - 0.20 micron depth of field for the visualisation of a 3 - 5 micron thick specimen create out-of-focus blur. This may still result in reasonable images for routine diagnostic microscopy, but it does not provide an image with an optimal light microscopic resolution. Making ultrathin sections (0.1 micron) for BFM is not a solution for this problem as such thin sections do not show enough absorption to be visible (Figure 7). Using an oil-immersion objective with a

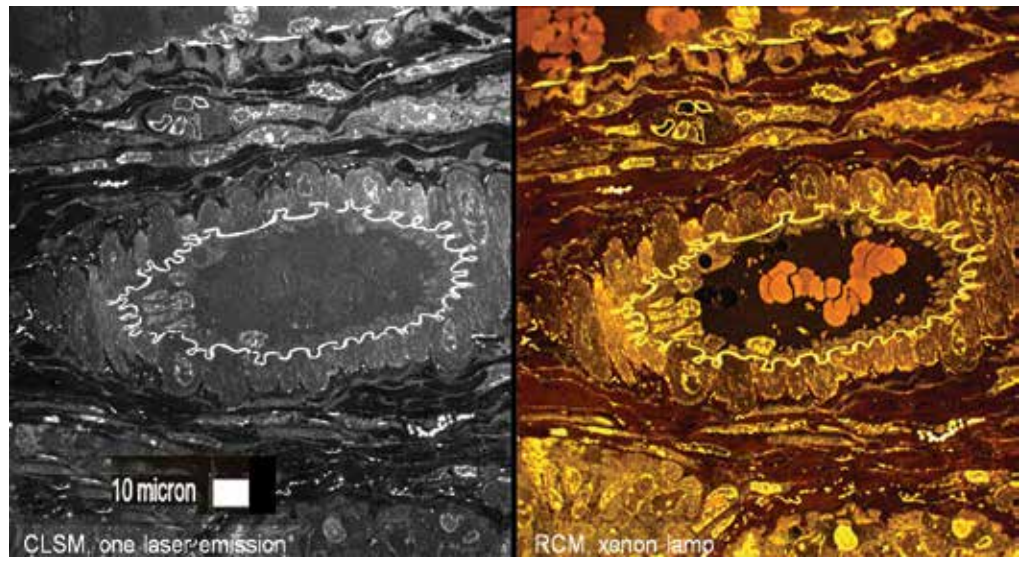

Figure 16. An image of a blood vessel obtained with light of one wavelength CRCM, results in a monochrome picture (left). The specimen stained with toluidine blue and illuminated with RCM using a Xenon high pressure lamp (emitting a rather broad colour spectrum), shows the multiple colours of the cytochemical staining (right)

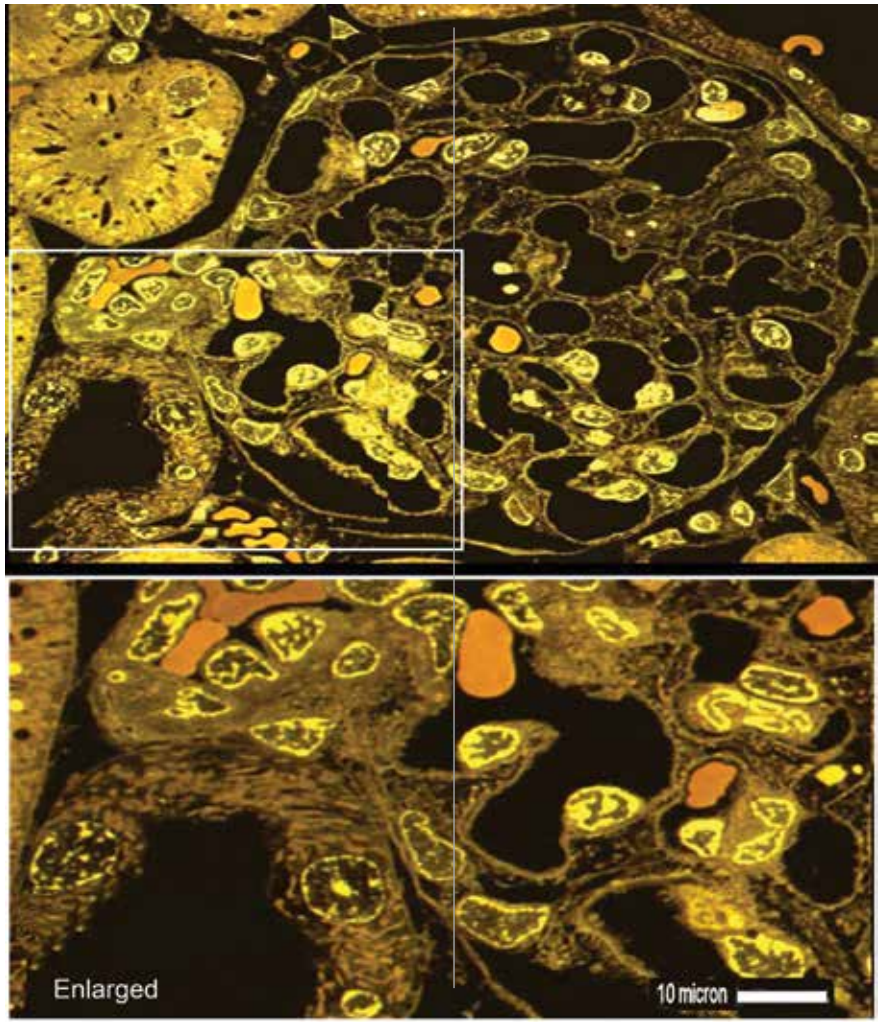

Figure 17. Conventional RCM of a ultrathin section of 0.1 micron produces images with brilliant colours, representing a broad spectrum when a high pressure xenon lamp is used. Toluidin blue staining

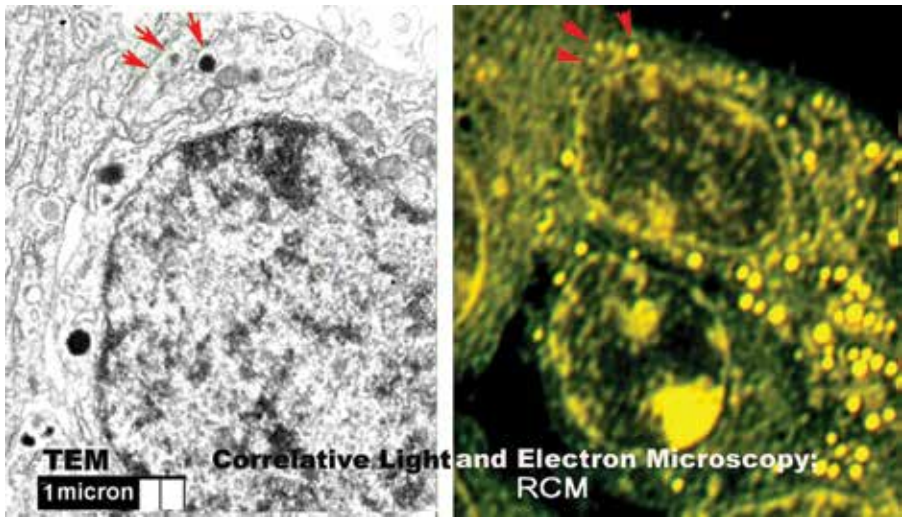

Figure 18. Correlative light and electron microscopy. Sequential ultrathin (80 nm) spurr sections. Sections observed with TEM (left) and RCM (right)

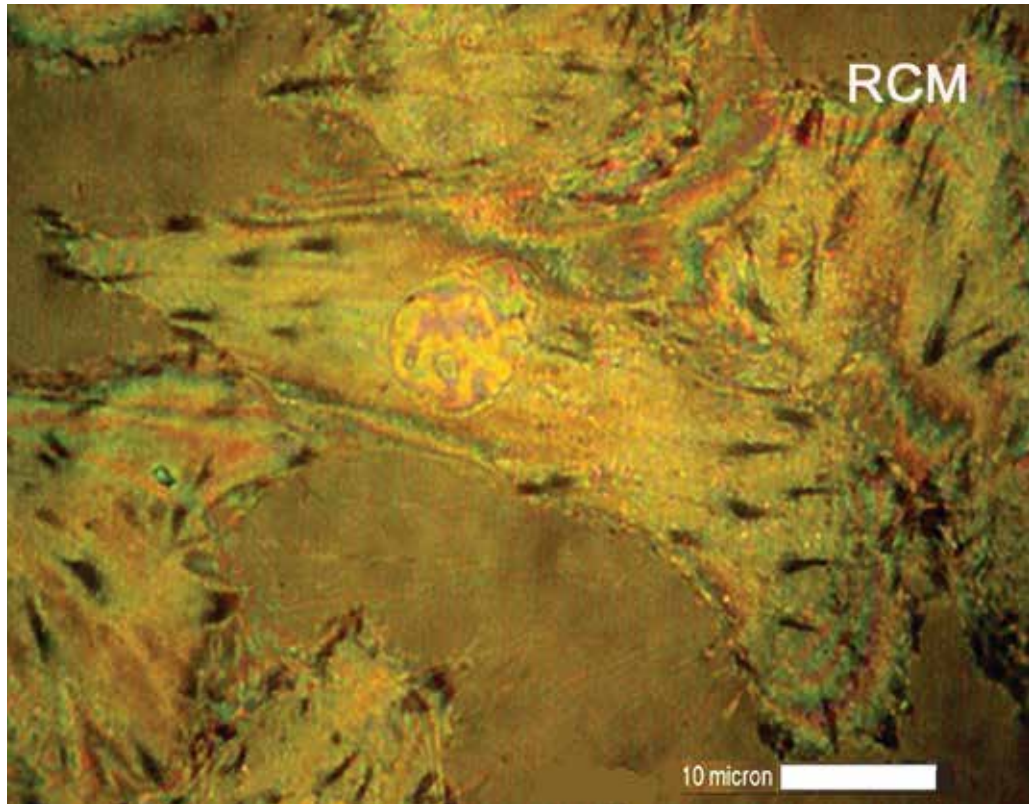

Figure 19. Culture of living cells growing on a glass surface. With RCM the zones of the adhesion of cells to glass are visible as dark areas

depth of field of about 0.15 - 0.20 micron, for RCM of 0.1 micron thick ultrathin sections, produces no out-of-focus blur. Such images show fine details with a high contrast and bright colours. These images could be described as "high definition" (Figures 13 & 14).

Several investigators have now reported regarding the high resolution obtained with RCM. Filler and Peuker 1997, describe an improved resolution obtained with RCM from peripheral nerve tissue. They noted that with the several types of conventional light microscopy systems they had used, they could not detect small nerve fibres reliably. If required, these features could be detected by evaluation of extensive areas by low magnification TEM, but this is a time consuming and expensive procedure. They concluded that with RCM satisfactory images from small nerve fibres could be obtained.

Confocal Reflection-Contrast Microscopy

Prins, 1993, (Department of Pathology, Leiden University) is the first person to have tested Confocal Reflection-Contrast Microscopy (RICM or CRCM, a proposed new term). With laser light of one wavelength monochromatic images can be obtained with confocal microscopy (Figure 15). Multicoloured images can be obtained by digital combination of several monochromatic confocal images. The use of conventional RCM for cytochemical staining can directly produce multiple colours (Figures 16 & 17).

Images obtained with confocal microscopy and RCM result in quite similar outputs since both do not suffer from out-of-focus blur.

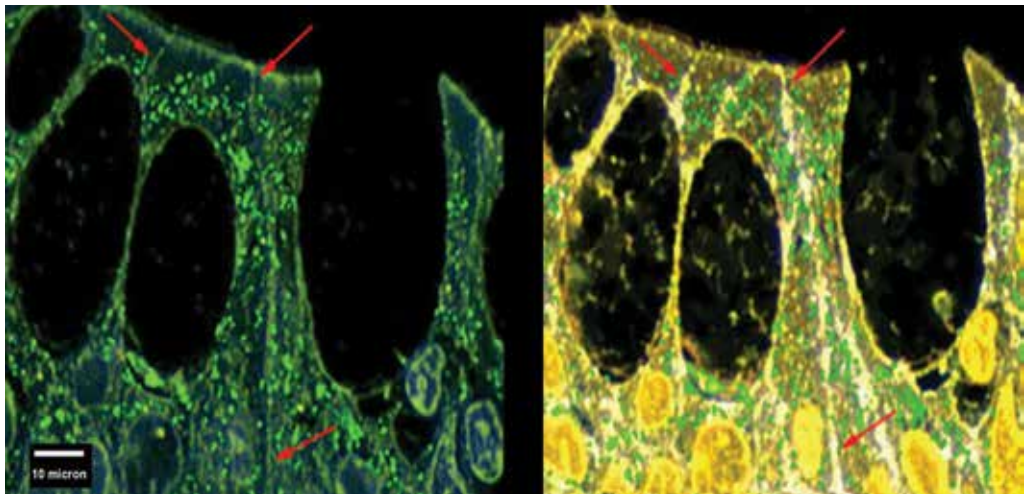

Figure 20. Double immunolabelling fluorescence (FITC) and immunogold silver of Pi-3 kinase (green spots) and Ep-CAM (red arrows) on 300 nm human colon cyro-section. Combination of RCM and FM. Fluorescence before counter-staining (left). Combined computer image counter-stained with Haematoxylin (right)

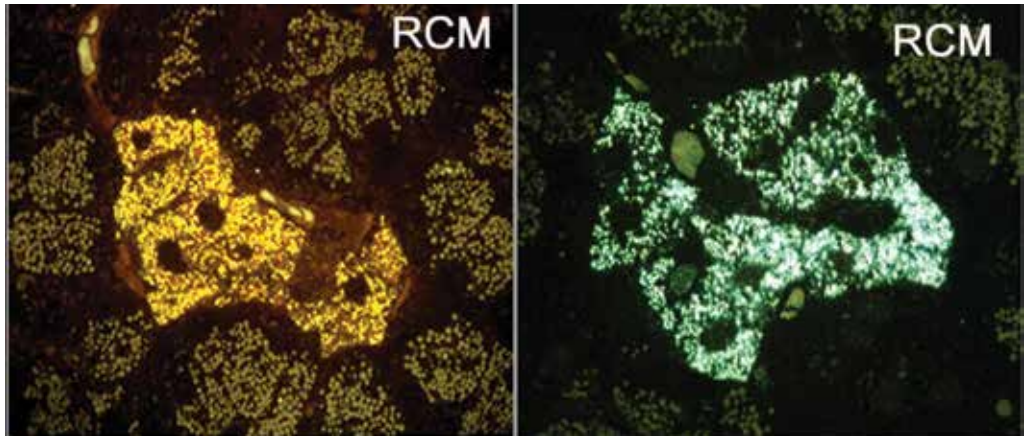

Figure 21. Ultrathin section of Langerhans islands in the pancreas. Immunostaining with 15 nm gold particles (left) and 5 nm gold (silver enhanced) particles (right)

Correlative light and electron microscopy

Versatile and interesting possibilities for correlative microscopy are offered by RCM. It can be carried out on a routine microscope equipped with the few devices needed for RCM. An ultrathin (80nm) plastic resin section can be stained with a variety of immunocytochemical methods. With RCM an image can be obtained with an optimal resolution, high contrast, no out-of-focus blur and the multiple colours of the cytochemical staining. A squential ultrathin 80 nm section placed on a grid can be examined with higher electron microscopy magnifications providing various resolutions (Figure 18).

RCM for the study of cell adhesion to glass, cell movement and cell shape

The study of adhesion of cells to a glass surfaces (Figure 19) was the first application area of RCM, (Ploem 1975). Bereiter-Hahn 1979, studied quantitative reflection contrast microscopy of living cells. He photographed images of cells at two different wavelengths (546 and 436 nm) and at two different angles of incidence. This allowed discrimination between reflected light and light that was both reflected and modulated by interference. Keller et. al. 1979, studied the locomotion of neutrophil granulocytes by RCM. They found that proteins as well as materials of low molecular weight have a marked effect on the rate of locomotion, adhesion and cell shape. Pera and Piper, 1980, reported on quantitative morphological analysis of erythrocytes by RCM. They investigated unstained and stained blood smears from normal individuals and patients with hereditary spherocytosis, sickle cell anemia, and thalassemia. The morphology of blood cells was extensively studied with RCM by Laumann, 1999 (Ph. D. thesis). Haemmerli, 1980, reported that RCM greatly facilitated the analysis of cellular motility. They investigated the activities of living cancer cells on glass substrates and observed stationary versus translocative motility and migration over or under other cells.

RCM and fluorescence microscopy

Hopman, et. al. 1986, described ISH that facilitated the simultaneous detection of different DNA sequences using Fluorescein Isothiocyanate (FITC) and Tetramethylrhodamine (TRITC) fluorescence marking to visualise different DNA target sequences in metaphase chromosomes. Some investigators used RCM as a limited replacement for BFM and phase-contrast microscopy A epi-illuminator equipped with filter cubes for BFM, RCM and FM can handle a large variety of biological and medical investigations (Figure 20).

RCM of immunogold and immunosilver staining

Hoefsmit, et.al., 1986, used RCM for the detection of gold particles after immunolabelling the plasma membrane of a macrophage-like cell line. Single 5 nm gold particles were clearly demonstrable with silver contrast enhancement and RCM. Cremers, et. al., 1987, tested the PO/DAB combination, either or not gold/silver intensified. Cornelese-ten Velde and Prins 1990a, also described the light microscopical detection of colloidal gold on ultrathin sections by RCM and were using ultra small gold particles with silver enhancement. They found an increased detection sensitivity of RCM compared with BFM. Hayat, 1995, mentioned in an extensive review publication, that good detection efficiency of immunogold and immunosilver staining can be obtained with RCM. He reported that gold particles (silver enhanced) as small as 5 nm can be visualised with this type of microscopy (Figure 21).

Ultrathin cryo-sections for RCM

The use of ultrathin cryo-sections enables the wide use of immunocytochemistry methods. Van Dorp, et. al., 1996, stated that RCM is a favourable method to visualise immunolabeling of ultrathin cryo-sections in comparison with BFM and FM. RCM has the extra advantage of also permitting the use of ultrathin sections for electron microscopy . Neelissen, et. al., 1999 described RCM for high resolution detection of 3H-estradiol in ultrathin cryo-sections of human stratum corneum. They concluded that RCM was as accurate as electron microscopy autoradiography (Figures 22 & 23).

Diagnostics of Alzheimer disease using RCM

Rickert, et. al. 1997, reported that RCM greatly facilitated their study of Alzheimer deposits of βA42-amyloid. In all studied cases RCM revealed capillaries with focal Aβ42 immunolabeling. Natté, et. al. 1999, investigated patients with hereditary cerebral hemorrhage with amyloidosis (Alzheimer Dutch type). Ultrathin sections were made and mounted on glass slides for RCM and on grids for electron microscopy. In all studied subjects, RCM revealed capillaries with focal immunolabelling. In the adjacent ultrathin electron microscopy sections (Aβ42) labelling was confined to the capillary basement membrane. Yamaguchi el.al. 2000, also used RCM to find the localisation of the diffuse plaques in ultrathin sections on glass slides. With TEM in sequential ultrathin sections on grids, the ultra structural localisation of amyloid β protein

(Aβ42) was observed in diffuse plaques (Figure 24). Kokubo, et. al. 2005, found that part of membrane-bound Aβ exists in rafts within senile plaques in the mouse brain. They confirmed that after localising the exact areas of senile plaques by RCM, these same plaques could be observed in corresponding locations with electron microscopy of sequential ultrathin sections.

Diagnosis of leptospirosis using RCM

Zhongxing, et. al. 1997, reported that RCM of a blood smear makes the detection of leptospires easy. Blood samples were collected from 37 cases. The authors suggested that RCM was an ideal method for diagnosis of leptospirosis. It was found to be simple, rapid and sensitive. (Reference in Chinese symbols).

Conclusions and outlook

Many researchers mention the high resolution of RCM images. This is in part due to the high contrast of RCM images. Resolution and image contrast are linked. High contrast results in a better resolution. The out-of-focus blur generated with BFM in routinely used 3 - 5 micron thick specimens reduces the resolution. This would explain the observed difference between routine BFM and RCM. Several investigators used RCM as a partial replacement for phase-contrast microscopy or BFM. It was mentioned that counter-staining of a specimen was often not needed with RCM. In some applications it permits the observation of images from microscopic objects that are difficult to be observed with other microscopic methods (Figure 25).

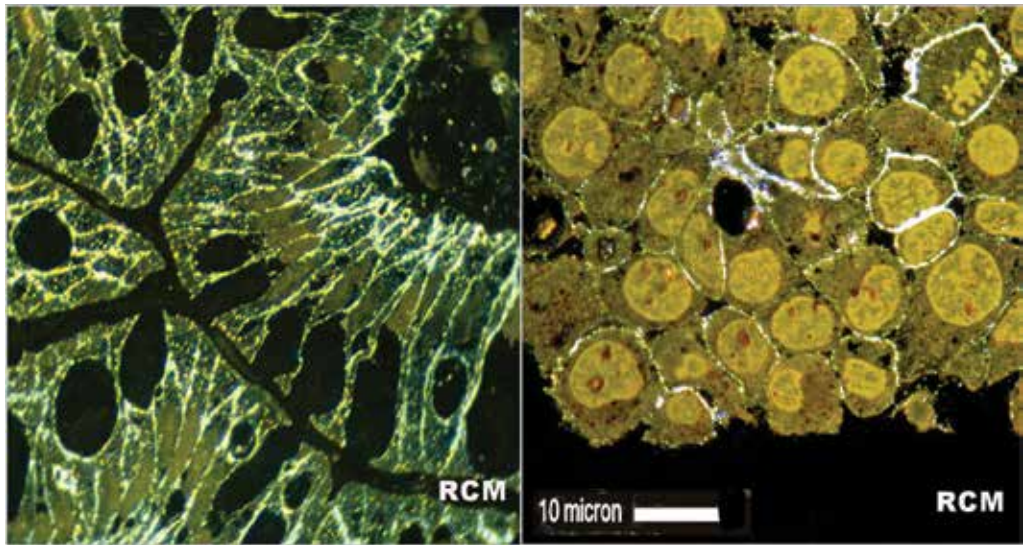

Figure 22. Immunohistochemistry of human colon (left) and cell aggregates (right) using ultra-cryo methods. Counter-stained with haematoxylin

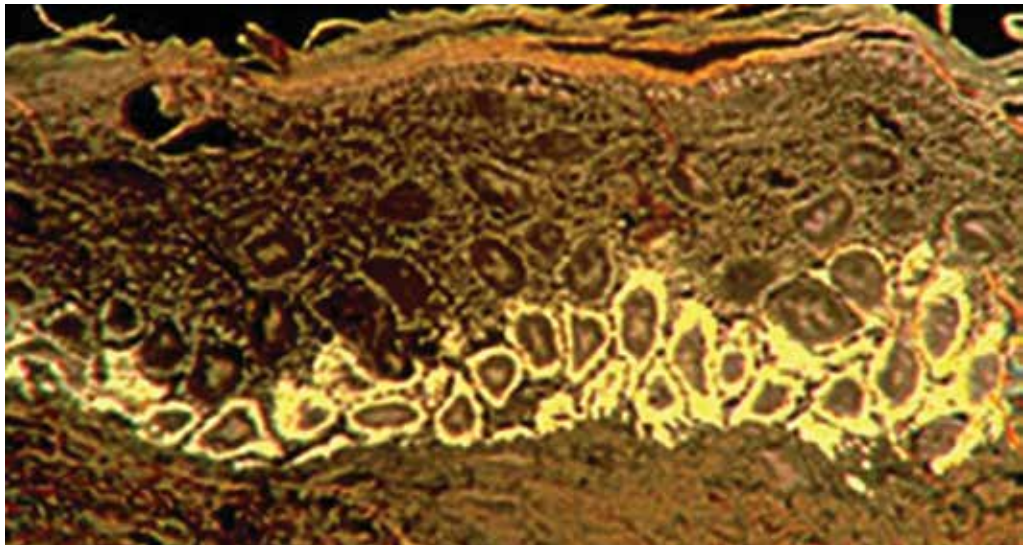

Figure 23. Ultrathin cryo-section (90 nm) of human skin stained with anti-keratin antibody peroxidase-DAB and not counter-stained

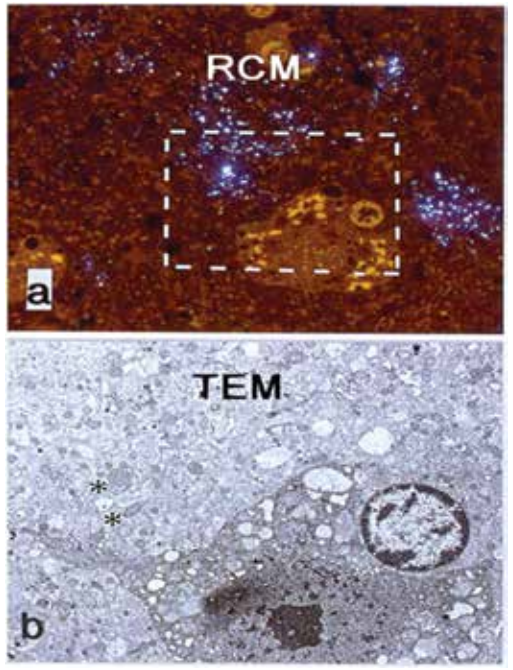

Figure 24. Dutch type Alzheimer disease. By using a low magnification search with RCM in the ultrathin sections of the (large) brain, it was possible to find the location of the Alzheimer amyloid plaques (top). Then for a more precise examination of the lesions, the adjacent (subsequent) ultrathin section was examined with TEM at a larger magnification (bottom)

The possibility to detect very small amounts of the DAB/peroxidase product after ISH was confirmed by several researchers. Ambros, et.al.1987, stated that the very sensitive detection technique with RCM presents a new approach for ISH procedures of chromosomes using biotinylated DNA probes. They also mentioned that in their opinion this method would in the future prove to be useful in a number of similar studies using other viral and oncogenic DNA probes. Interestingly, they succeeded in the chromosomal insertion of human papillomavirus 18 sequences in HeLa cells, using RCM. Speel, et.al.,1993, have developed a method for the detection of different cytochemical reaction products by means of RCM. They concluded that RCM is a sensitive method for the visualisation of multiple targets in biological specimens. Many cytochemical stains have already been used with RCM, presenting polychrome images for the identification of different cellular components. This use is likely to continue in the future, using more and different cytochemical markers including

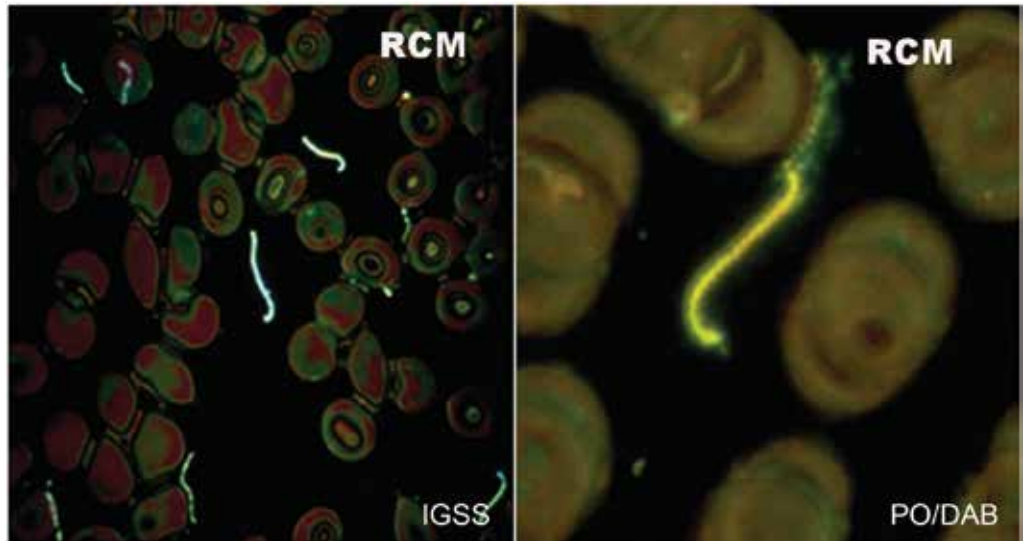

Figure 25. With RCM the reflection of light by leptospires in a bloodsmear makes their detection easily possible. Using FM the identification of leptospires is much more difficult

combinations with silver and gold particle markers. The fact that the epi-illuminator of a RCM microscope also allows the introduction of filter cubes for fluorescence microscopy, will enable the development of new multi target combinations of cytochemical markers. The identification of DNA sequences on the tiny genes of a chromosome using ISH with RCM may be considered as a step towards super sensitive light microscopy. Takashima et. al., 2001, stated that very sensitive detection methods are desperately needed if the great potential of molecular genetics in clinical practice has to be realised.

Maybe other very sensitive microscope techniques are already being investigated. But at present, RCM is available as a super sensitive light microscopy method for this type of gene research. Prins et. al., 1993, have stated that RCM offers a bridge between light and electron microscopy. A large number of researchers has already used this possibility. In many cases they used the RCM of ultrathin specimens to find rare lesions in large organs (e.g. the brain) at a lower light microscopic magnification. In corresponding subsequent ultrathin sections of the same specimen they could then verify the type of lesion using higher electron microscopy magnifications. Using electron microscopy alone would have been more time consuming for locating rare lesions. For researchers who don't have a confocal microscope available to obtain microscope images with no out-of-focus blur, there is the interesting possibility to visualise for first time ultrathin sections with a thickness of about 0.1 micron with RCM. This produces sharp and bright images with a high contrast and no out-of-focus blur. Since alternate (subsequent) ultrathin sections of the same specimen can be examined with electron microscopy, correlative microscopy can be easily realised.

In summary there are 5 factors that have made RCM popular: [1] High contrast images. [2] Increased microscope resolution of images. [3] No out-of-focus blur when using ultrathin sections. [4] Super sensitivity for the detection of minute amounts of some cytochemical reaction products. [5] An easy possibility for correlative light or electron microscopy.

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