Technical Advance

Comparison of Different Detection Methods in Quantitative Microdensitometry

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Quantitative evaluation of immunohistochemical staining has become a focus of attention in research applications and in pathological diagnosis, such as Her-2/neu assessment in mammary carcinoma. Reproducibility of immunostaining techniques and microscopical evaluation are prerequisites for a standardized and reliable quantitation of immunostaining intensity. In the present study, different staining and microscopical techniques, including fluorescence microscopy, epipolarization microscopy of immunogold-silver, and absorbance microdensitometry were compared concerning suitability for quantitative evaluation. We describe a staining procedure using alkaline phosphatase-based immunohistochemistry with the substrate Vector Red and subsequent microdensitometry with a custom-designed absorbance filter. We have characterized linearity of the staining intensity in dependence of development time, antibody concentration, and section thickness by means of artificial standards consisting of agarose blocks into which immunogold- or alkaline phosphatase-conjugated antibodies were incorporated. Applicability of the different techniques was tested by anti-CD45 immunostaining of leukocytes within rat lung tissue detected by immunofluorescence, immunogold-silver epipolarization microscopy, as well as alkaline phosphatase-based Vector Red absorbance or fluorescence measurement. Excellent qualities of Vector Red for quantitative microdensitometric evaluation of staining intensity were particularly obvious for absorbance microscopy. Applicability in paraffin-embedded tissue as well as in cryosections, excellent segmentation, linearity over a wide range, light stability, and feasibility for permanent mounting and for long-term storage are the outstanding features of this technique

for use in routine quantitative evaluation. (*Am J Pathol* 2001, 158:407–417)

Cellular localization of target substances by immunohistochemistry is a widely used technique and an important tool in research as well as in histopathological diagnosis.^{1–3} Qualitative evaluation is, for example, primarily used for the investigation of regulatory processes.^{4–6} During the last few years, quantitative evaluation of staining intensity has attracted growing interest in research and diagnosis in the field of pathology. Microdensitometric quantitation has been used in studies investigating breast cancer for proliferation signals and steroid receptor expression,^{7–9} as well as in prostate cancer specimens for expression of prostate-specific acid phosphatase.¹⁰ In recent studies, Her-2/neu, a proto-oncogene, which encodes a growth factor receptor of the tyrosine kinase family, has been shown to be overexpressed in 25 to 30% of breast and ovarian malignancies.^{11,12} Quantification of Her-2/neu expression or its encoding protein has become an important diagnostic tool for prognosis assessment and prediction of response to targeted gene therapy.^{12,13} *In situ* hybridization and immunohistochemistry have both been shown to be powerful techniques for the evaluation of Her-2/neu expression.^{11,12}

Numerous techniques used for quantification of mRNAs or proteins are used for investigation of tissue homogenates, thus delivering an averaged result without relation to specific cell types and cellular environment. Immunohistochemistry or *in situ* hybridization in combination with digital image analysis provide both *in situ*-localization and objective observer-independent quantitative evaluation.⁴ Several techniques of staining and detection for quantitative immunohistochemistry have been described that use enzyme-dependent color reactions as well as fluorescence or epipolarization methods.^{5,14–16} Quantification of immunogold-stained targets was predominantly used in electron-microscopic studies.^{17–19}

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Only a few reports have hitherto addressed the quantification of immunogold-silver-enhanced staining intensity by epipolarization imaging in light microscopy.^{20,21} This group recently described quantitative evaluation of Cox-1 and Cox-2 immunostaining by image analysis of immunogold-silver-enhanced staining intensity by epipolarization imaging.^{22,23} A similar technique, the reflection contrast microscopy, has frequently been used for detection and quantification of the peroxidase product diaminobenzidine (DAB),^{24–27} but requires the use and disposal of the toxic substrate DAB. In bright-field microscopy, absorbance techniques can be applied without expensive microscopic equipment as is usually required for fluorescent techniques. Absorbance techniques, which measure the optical density of enzymatic reaction products, have been described for the use of peroxidase- and alkaline phosphatase-linked detection systems.^{5,28} Absorbance measurement of the peroxidase substrate DAB or the alkaline phosphatase substrate Fast Red has been used in several approaches to determine antigen concentrations.^{29–33} In addition, several approaches of standardization have been made to prove a quantitative correlation of staining intensity and antigen or antibody concentration.^{34–39}

Independent of the method used, standardization of the technical procedure is crucial for obtaining accurate and reliable results. Moreover, standardization is needed to meet the requirements of quality control concerning objectivity and reproducibility.^{6,28,35,40} An additional prerequisite in diagnostic pathology is the necessity for stability of staining and permanent mounting for long-term storage of sections to provide documentation and verification of the diagnosis.

Here, we present the use of the substrate Vector Red for alkaline phosphatase-linked immunostaining and microdensitometry. Vector Red provides a stable bright red precipitate visible in bright-field and fluorescence microscopy, and it offers the possibility of permanent mounting and long-term storage. In addition, we compared different detection techniques (epipolarization, fluorescence, and absorbance) for quantitative evaluation of staining intensity. Standards for both immunogold- and alkaline-phosphatase-based techniques were created by means of agarose blocks into which antibodies coupled with either ultra-small gold particles or alkaline phosphatase were incorporated. The linearity of staining intensity was characterized by using different antibody concentrations, sections of different thickness, and increasing development time. In essence, the amount of precipitate or dye (silver grains or Vector Red) was found to be linear with antibody concentration, thickness of sections, and development time, thus qualifying both detection methods as appropriate for quantitative analysis of light-microscopic immunohistochemistry, offering several advantages in comparison with common staining and detection techniques.

Materials and Methods

Reagents

Agarose low electroendosmosis was obtained from Boehringer Mannheim (Mannheim, Germany). Chicken

egg white was provided from Chroma (Münster, Germany), bovine serum albumin, poly-L-lysine, methyl green, nuclear Fast Red, Mayer's hematoxylin, the alkaline phosphatase substrate Fast Red and 4', 6-diamidino-2-phenylindole (DAPI) were obtained from Sigma (Deisenhofen, Germany). Paraffin liquid (no. 107162) and low-temperature paraffin (no. 107150) were both obtained from Merck (Darmstadt, Germany).

Anti-CD45 antibody (no. MCA-43G, clone MRC OX-1) was purchased from Serotec (Oxford, UK). Secondary antibodies goat-anti-mouse (alkaline phosphatase-conjugate, Texas Red-conjugate, ultra-small gold-conjugate) were provided by Nanoprobes (Stony Brook, NY) and Biotrend (Cologne, Germany). The silver enhancement solution was acquired from Aurion (Wageningen, The Netherlands). The alkaline-phosphatase substrate kit Vector Red was purchased from Vector Laboratories (Burlingame, CA). Clarion and Crystallmount were purchased from Natutech (Frankfurt, Germany), and Permafluor was provided by Immunotech (Hamburg, Germany).

Preparation of Standard Blocks

Agarose LE was dissolved in boiling double-distilled water resulting in a 4% agarose solution. During constant stirring, the solution was cooled down to 42°C. Meanwhile, the antibody was admixed in different concentrations (AP-conjugated antibody 1:125, 1:250, 1:500, colloidal gold-conjugated antibody 1:50, 1:100, 1:200, 1:400) to a solution of 5% bovine serum albumin and 10% egg white in double-distilled water. The antibody-containing solution was then added 1:1 to the agarose solution and thoroughly mixed. Subsequently, the mixture was aspirated in a 1-ml syringe and rapidly cooled down at 4°C to accelerate gelling of the agarose. The solid standard block was removed from the syringe and cut into 3- to 5-mm-sized cylinders.

Paraffin-embedding was performed by dehydration of the blocks in a graded series of alcohol and immersion in a mixture of 6:4 low-temperature paraffin and paraffin oil at 40°C overnight.

Sectioning of 5, 10, 15, and 20 μm was performed in a cryostat (CM 3050; Leica, Benzheim, Germany) at -25°C . Sections were placed in cold water and mounted on poly-L-lysine and chicken egg white-coated glass slides.

Staining of Standard Sections

The sections were dewaxed, rehydrated, and washed in double-distilled water for 5 minutes and sections with incorporated AP-labeled antibody were incubated with Vector Red substrate for different periods (10 minutes to 60 minutes at intervals of 10 minutes) in a humid chamber. For development the slides were placed in the dark, at a constant temperature of 20°C in an incubator (Heraeus Instruments GmbH, Hanau, Germany). For bleaching experiments sections of the AP-labeled standard blocks were additionally developed with the AP-substrate Fast Red for 40 minutes.

Sections of immunogold standards were incubated with silver enhancement solution for 40 minutes under constant conditions in the same incubator. The incubation was then rapidly interrupted by thoroughly rinsing the sections in double-distilled water. Negative staining controls for background subtraction were made without incubation of substrate solution or silver enhancement solution. Subsequently, the sections were dehydrated and permanently mounted.

Animal Preparation

The rats (male; body weight, 350 to 400 g) were deeply anesthetized with sodium pentobarbital (100 mg/kg body weight intraperitoneally). After local anesthesia with 2% xylocaine and median incision, the trachea was dissected and a tracheal cannula was immediately inserted. A median laparotomy was performed and subsequently the rats were anticoagulated with 1,000 U of heparin. After midsternal thoracotomy, the right ventricle was incised, a cannula was fixed in the pulmonary artery, and the apex of the heart was cut off to allow pulmonary venous outflow. Simultaneously, pulsatile perfusion with buffer solution was started. The buffer contained 2.4 mmol/L CaCl_2 , 1.3 mmol/L MgCl_2 , 4.3 mmol/L KCl, 1.1 mmol/L KH_2PO_4 , 125.0 mmol/L NaCl, 25 mmol/L NaHCO_3 as well as 13.32 mmol/L glucose (pH ranged between 7.35 and 7.40). The lungs were continuously perfused for ~5 minutes for washout of blood.

Tissue Preparation

Lung tissue was chosen as test tissue for this study, because its high content of elastic fibers makes it a difficult object for evaluation, eg, in fluorescence microscopy. Fixation was performed by tracheal instillation of a 3% paraformaldehyde solution at a pressure of 20 cm H_2O and immersion of the lungs in the same fixation solution for 1 hour ($n = 5$, each group). For paraffin embedding the entire lung was dissected into tissue blocks from all lobes. The tissue blocks were embedded in low-temperature paraffin with a melting temperature of 40 to 42°C. Sectioning of 10 μm thickness was performed from all paraffin blocks on a paraffin microtome (Reichert-Jung Histoslide 2000; Cambridge Instruments, Nussloch, Germany).

In addition lungs were instilled with TissueTek OCT compound and snap-frozen in liquid nitrogen, dissected in tissue blocks from all lobes, and stored at -80°C. Sectioning of 10 μm thickness was performed from frozen tissue blocks of both left and right lungs in a cryostat (CM 3050; Leica, Benzheim, Germany).

Immunohistochemistry

Immunogold-Silver Staining

The paraffin sections were dewaxed, rehydrated, and washed in phosphate-buffered saline (PBS) (0.01 mol/L, 150 mmol/L NaCl, pH 7.6) for 3×5 minutes. Cryosec-

tions were fixed in a 3% paraformaldehyde solution for 5 minutes and washed in PBS 3×5 minutes. The sections were pre-incubated in PBS containing 5% goat serum, 1% bovine serum albumin (BSA), 0.1% BSA-C, 0.05% Tween-20, and 0.02 mol/L glycine to block nonspecific binding. Overnight incubation with the monoclonal anti-CD45 antibody diluted 1:100 in PBS containing 1% BSA, 0.1% BSA-C, and 0.05% Tween-20, was performed at 4°C. The sections were then washed in PBS and incubated with the secondary gold-conjugated antibody diluted 1:400 overnight at 4°C. Next the sections were washed in PBS again and fixed for 5 minutes in 2% phosphate-buffered glutardialdehyde. After several washes in glass-double-distilled water the sections were incubated in Silver Enhancer solution for 40 minutes. Counterstaining of the sections was performed with nuclear Fast Red.

Alkaline Phosphatase Staining

Paraffin and cryosections were processed accordingly. The sections were pre-incubated in PBS containing 5% goat serum, 1% BSA, and 0.05% Tween-20 to block nonspecific binding. Incubation conditions with the primary anti-CD45 antibody (1:100 in PBS containing 1% BSA and 0.05% Tween-20) were as described above. The secondary AP-conjugated antibody (dilution, 1:4,000) was incubated in the same dilution buffer overnight at 4°C. Afterward, washing of the sections in PBS for 3×5 minutes was performed. Subsequently, sections were developed with a Vector Red Substrate Kit for 30 minutes. Levamisole, 2.5 mmol/L, was added to inhibit endogenous AP activity. Counterstaining of these sections was performed with methyl green.

Texas Red Staining

Cryosections were fixed in a 3% paraformaldehyde solution for 5 minutes and washed in PBS for 3×5 minutes. The sections were preincubated and treated with primary antibody as described in Alkaline Phosphatase Staining. The secondary Texas Red-conjugated antibody was applied in a concentration of 1:1,000 overnight at 4°C. Counterstaining was performed with Mayer's hematoxylin or DAPI.

Control staining was performed by omission of the primary antibody and substitution with nonspecific serum at the same dilution.

Mounting

Immunogold and AP-Vector Red-stained sections were permanently mounted with Clarion and coverslips, whereas Texas Red-labeled sections were mounted in an aqueous mounting medium (Permafluor). AP-Fast Red-stained sections were mounted without coverslips with the water-soluble Cyralmount, which hardens like a permanent mounting medium and can be visualized with oil immersion.

Image Analysis

An image analysis system with a 12-bit cooled charge-coupled device camera (Sensys KAF 1400; Photometrics, Tucson, AZ) mounted on a fully automated Leica DM RXA (Leica, Wetzlar, Germany) was used to digitize gray-scale images to a Dual-Pentium 200 MHz host computer. The 12-bit camera has a high dynamic range with a nonlinearity lower than 0.5%. Microscope settings were kept constant throughout all measurements (objective, 40 $\times$ oil; Leica PL Fluotar 40 $\times$ /0.75). For epipolarization and fluorescence imaging a stabilized 12-V high-pressure mercury-vapor lamp, 100 W, was used for illumination. A Leica IGS epipolarization filterblock (POL no. 513813) and a Texas Red filterblock (TX2 no. 513843) were used for either epipolarization or fluorescence microscopy. Absorbance measurement was performed with a custom-designed filter for absorbance of the substrate Vector Red (central wavelength, 525 nm; half band width, 10 nm $\pm$ 2 nm) in optical quality (manufactured by Omega Optical, Inc., Brattleboro, VT). The optimal central wavelength was determined by measurement of the substrate in a Leica MPV SP Microscope Photometer System by courtesy of Leica. Estimation of section thickness by detection of autofluorescence was performed with a fluorescein isothiocyanate filter. A stabilized 12-V tungsten-halogen lamp, 100 W, was used for illumination. Bright-field images were taken with a Leaf Microlumina (Scitex Corporation Ltd., Herzlia B, Israel), mounted on the 35-mm photo exit of the Leica DM RXA.

Adjustment of all microscope settings was stored and recalled before measurement. Calibration of the measurement system with a reference slide was done before measurement. Gray-scale images were digitized to 12 bit accuracy resulting in an intensity scale ranging from 0 to 4095. Image analysis was performed by means of the image analysis program ImagePro 3.0 (Media Cybernetics, Del Mar, CA). For direct visualization of staining intensity a pseudocolor scale with 11 colors was chosen, each representing an equal sector of the intensity scale, and applied to the images. Background measurement was performed to evaluate the influence of nonspecific antibody binding.

Measurement Procedure

From the standard blocks at least three sections of each thickness (5, 10, 15, and 20 μ m) and each staining time (intervals of 10 minutes) were measured (gain 1, exposure time: Vector Red, 400 ms; IGSS, 20 ms). From each section 5 images (1024 $\times$ 1024 pixels) were digitized. Data of integrated optical density and area of each image were automatically measured and subsequently transferred into the spreadsheet program EXCEL 7.0a (Microsoft Corporation, Redmond, WA). CD45-immunostained alveolar macrophages were likewise selected in an area of interest and automatic measurement was performed from 5 to 10 sections of each different immunostaining technique.

For investigation of photobleaching of Vector Red, Fast Red, Texas Red, and immunogold-silver in absorbance,

epipolarization, or fluorescence detection intensities, images from at least three different areas per section were digitized and intensity was measured. At certain intervals of permanent light exposure (every 0.5 minute during the first 10 minutes, after 15, 30, and 60 minutes, after 24 hours) the signal intensities were measured.

Autofluorescence for quality control of section thickness was measured from eight randomly chosen regions of at least three unstained sections. Detection of the autofluorescence signal was performed at 480 $\pm$ 40 nm excitation and 527 $\pm$ 30 emission (fluorescein isothiocyanate filter, L5 no. 513840; Leica). The autofluorescence signal was rather low, so the detection had to be optimized with higher camera gain (gain 3) and exposure time (8 seconds).

Statistical Analysis

Analysis of variance was used to evaluate differences among different groups. A value of $P < 0.05$ was considered significant. Data are given as mean $\pm$ SEM.

Results

Segmentation

Segmentation of signal in immunogold-silver staining was achieved by epipolarization microscopy using a special epipolarization filter. Epipolarization depiction of the silver-enhanced colloidal gold particles created a complete segmentation between positively stained and unstained tissue.

The individual transmission spectra of the substrates Vector Red, and the counterstains methyl green and Mayer's hematoxylin are shown in Figure 1. Vector Red staining was measured at its absorbance maximum at a wavelength of 525 $\pm$ 10 nm (Figure 1A). Methyl green was found to be the best-fitting counterstain for Vector Red signal segmentation because of its absorbance maximum at 660 nm (Figure 1B), which did not overlap with Vector Red. Mayer's hematoxylin in contrast was

Figure 1. Transmittance curves of Vector Red, methyl green, and Mayer's hematoxylin. Note that the absorbance maxima of Vector Red and methyl green do not overlap. Central wavelength (CWL) was chosen in the absorbance maximum of Vector Red to be 525 nm for the custom-designed absorbance filter.

slightly visible at 525 nm, because of its wide range of absorbance (Figure 1C).

In fluorescence microscopy both Vector Red and Texas Red staining were detected at >615 nm. Segmentation was, however, limited by autofluorescence of the tissue, which may be rather strong in a variety of organs, eg, lung tissue because of the abundance of elastic fibers.

Standard Agarose Blocks

Embedding of agarose blocks in paraffin and lowering of the temperature were necessary for precise sectioning of the standard blocks. Accuracy of section thickness was assessed by measuring the extent of autofluorescence, to which it is tightly correlated.⁴¹ In addition, to minimize the impact of section thickness on measurement accuracy at least three sections for each measurement were used.⁴² The antibodies were found to be homogeneously distributed within the agar matrix (Figure 2). Silver-enhancement of colloidal gold occurs by deposition of metallic silver, which clusters around colloidal gold particles and produces a grain-like appearance (Figure 2A). Staining intensity, however, was determined by integration of staining intensity over a complete microscopic field of $36,503.62 \mu\text{m}^2$. Alkaline-phosphatase-related staining in contrast resulted in a homogeneous distribution with equal intensity over the complete field (Figure 2B, inverted image).

The linear range of both absorbance and fluorescence of Vector Red was assessed throughout a development period of 60 minutes (Figure 3). Absorbance of Vector Red proved to be linear throughout the entire period, thus allowing quantitative evaluation at any staining time up to 60 minutes, which may be necessary when adjusting an immunohistochemical protocol for detection of an antigen. Fluorescence of the substrate Vector Red was detectable with a Texas Red filter, which displays a bright-red fluorescence signal at >615 nm. Fluorescence measurement of Vector Red signal resulted in a nonlinear curve with a linear range between 20 and 40 minutes.

standard blocks

Figure 2. Artificial standard agarose blocks. **A:** Antibodies conjugated with ultra-small gold particles (concentration, 1:50; silver enhancement, 40 minutes; epipolarization image). **B:** Antibodies conjugated with alkaline phosphatase 1:125, Vector Red development 10 minutes, absorbance with custom-designed filter, inverted image. Note the homogeneous distribution of antibodies within the agar blocks.

Linearity of VectorRed signal in dependence of development time

Figure 3. Comparison of the linearity range of Vector Red absorbance and fluorescence measurement. Absorbance measurement was linear throughout 60 minutes of substrate development, whereas fluorescence measurement was only linear within a short range of 20 to 40 minutes of substrate development. Antibody concentration 1:250, 10 μm section thickness.

Absorbance measurement was linearly related to different antibody concentrations admixed to the agar blocks (Figure 4A). There was also a good correlation between the thickness of sections and staining intensity, as determined by absorbance measurement (Figure 4B). Linear correlation between section thickness or antibody concentration with staining intensity was also determined for Fast Red development. In epipolarization imaging of immunogold-silver stained sections of agarose blocks, a linear relationship between antibody concentration and staining intensity was similarly demonstrated (Figure 5).

Stability of Staining

The stability of the staining intensity at light exposure was examined throughout a 1 hour period (Figure 6) and after 24 hours. A considerable bleaching of the staining was noted for the fluorescent dye Texas Red (Figure 6), but was also detected to a minor extent with the alkaline phosphatase substrates Vector Red and Fast Red in fluorescence microscopy (Figure 6, Table 1). Absorbance measurement of Vector Red and Fast Red staining revealed stable intensities throughout a 1 hour period, but no significant change was observed throughout an observation period of 24 hours. Similarly, absorbance and epipolarization of immunogold-silver staining was entirely stable, without showing any fading throughout 24 hours (Figure 6).

Background

Development of alkaline phosphatase with Vector Red produces a bright-red precipitate, which is clearly detectable by absorbance with the described custom-designed filter. Nonspecific staining and background staining was low, both in paraffin-embedded tissue and cryosections. Staining was absent from sections where the primary antibody was omitted or nonspecific serum was applied.

Absorbance measurement of VectorRed signal - Characterization using standard blocks

Figure 4. Linearity of Vector Red absorbance in relation to antibody concentration (A) and section thickness (B). Thickness of section, 10 μ m (A); antibody concentration, 1:500 (B).

In contrast, fluorescence microscopy of Vector Red- and Texas Red-stained tissue is hampered by tissue-specific autofluorescence, which is extremely strong, eg, in lung tissue (Table 1).

Immunogold staining with subsequent silver enhancement showed excellent results in paraffin-embedded tissues. Positive-stained structures were unambiguously detectable and background staining was low. On use in cryosections, in contrast, immunogold-silver staining may exhibit nonspecific and background staining. For this reason, the segmentation of stained structures in epipolarization primarily depends on the antibody used. Appli-

cation of epipolarization in pathological human tissue is limited by foreign bodies/material, eg, anthracotic pigment deposition in the lung, which shows epipolarization and may hinder segmentation and determination of positively stained structures (Table 1).

Application

CD45 immunostaining was performed on differently embedded tissue and with different immunostaining protocols. Immunogold-silver staining (Figure 7, A-C) resulted

Epipolarization of Immunogold-Silver signal - Characterization using standard blocks

Figure 5. Linearity of immunogold-silver epipolarization intensity in relation to antibody concentration; thickness of section, 10 μ m.

Fading characteristics of different dyes in dependence of light exposure

Figure 6. Comparison of photostability of various dyes using different microscopic techniques (epipolarization, absorbance, and fluorescence). Immunogold-silver was measured with epipolarization and absorbance detection. Vector Red and Fast Red were measured using absorbance and fluorescence imaging. Texas Red was measured using fluorescence microscopy. Photobleaching was evident in fluorescence microscopy especially when using the fluorescent marker Texas Red.

Table 1. Summary Table

Features	Immunogold-silver enhancement staining	Alkaline phosphatase-dependent staining		Fluorescent staining label: Texas Red
		Substrate: Vector Red	Substrate: Fat Red	
Counterstain	Nuclear Fast Red, methyl green excellent contrast	Methyl green, Mayer's hematoxylin excellent contrast	Mayer's hematoxylin excellent contrast	Fluorescent dye: eg, DAPI or Mayer's hematoxylin
Mounting	Permanent with coverslips	Permanent with coverslips	Aqueous, nonpermanent; permanent without coverslips (Crystalmount)	Aqueous, non-permanent
Microscopic detection	Bright field or epipolarization	Bright field or fluorescence	Bright field or fluorescence	Fluorescence
Technical equipment for quantitation	Epillumination and epipolarization filter, Customary camera is sufficient, possibly stabilized light source	Common bright field and absorbance filter, Customary camera is sufficient, possibly stabilized light source	Common bright field and absorbance filter, Customary camera is sufficient, possibly stabilized light source	Epillumination fluorescence filters and objectives, light-sensitive camera with light shutter stabilized mercury-vapor lamp
Background	Paraffine sections = low cryosections = considerable	Low	Low	Considerable eg, autofluorescence or out of focus signal
Nonspecific signals	Foreign body material eg, anthratic pigmentation	None, but endogeneous AP activity needs sufficient inhibition with levamisole	None, but endogeneous AP activity needs sufficient inhibition with levamisole	Tissue-dependent autofluorescence eg, elastic fibers
Linearity of signal	Epipolarization: large linear range	Absorbance: large linear range (fluorescence: low linear range)	Absorbance: large linear range (fluorescence: low linear range)	Low linear range
Light stability	In bright field and epipolarization stable	In bright field and absorbance stable (in fluorescence slight photobleaching)	In bright field and absorbance stable (in fluorescence considerable photobleaching)	Extensive photobleaching
Accuracy of measurement	Low variability, measurement indefinitely repeatable because of light stability	Low variability, absorbance measurement indefinitely repeatable because of light stability	Low variability, absorbance measurement indefinitely repeatable because of light stability	High variability, immediate measurement required, not repeatable because of photobleaching
Storage	Permanent storage	Permanent storage	Aqueous: no permanent storage Permanent: without coverslips	No permanent storage

in a strong and clear signal, which showed good contrast to the counterstain nuclear Fast Red in bright-field microscopy (Figure 1A). Epipolarization created a complete segmentation between stained and unstained structures (Figure 7B). Different staining intensities can be visualized using a pseudocolor depiction, which represents different gray-scale ranges (Figure 7C). Vector Red staining gives best contrast with methyl green counterstaining (Figure 7D), which is not visible using the custom-designed absorbance filter (Figure 7E).

Texas Red immunofluorescence gives sufficient staining results (Figure 7G), which, however, can only be visualized using expensive fluorescence microscopy equipment. Intensity measurement has to be performed immediately to prevent photobleaching. Large measurement deviations can be minimized with an automated microscope equipped with a light shutter, but photobleaching is unavoidable by this approach because structures have to be searched for extended time periods. Intensity measurement of CD45-stained alveolar macrophages showed minimal scattering with immunogold-silver epipolarization ($SEM \pm 7.9\%$ of mean values) and Vector Red absorbance measurement ($\pm 8.9\%$). Fluorescence measurement using Texas Red and Vector Red resulted in higher variability (Vector Red, 15.3%; Texas Red, 13.3%), because of the influence of fading.

Discussion

During the past few years, computer-assisted image analysis has been increasingly used for quantitative evaluation of histopathological and cytopathological features in the research areas and in diagnostic pathology.^{1,3,40} The quantification of the final reaction product of immunohistochemistry has been addressed in numerous studies, and different staining techniques were evaluated with respect to standardization and linearity, prerequisites for obtaining reliable and reproducible quantitative results.^{5,28,39} Such standardization also includes technical steps, therefore the influence of sectioning, antibody concentration, and staining time were evaluated in this study. To perform a comparison of different methods in quantitative microdensitometry, we created artificial test blocks by incorporation of antibodies labeled with either immunogold or alkaline phosphatase into agarose, and this standard specimen served for characterization of the immunogold-silver and Vector Red-staining intensity.

There have been previous efforts to create artificial test standards for the characterization of reaction products, eg, the peroxidase substrate DAB, by incorporation of antigens or antibodies in agarose or gelatin media or coupling them with Sepharose beads.^{32,34,43} Linearity between DAB absorbance and concentration or staining

Figure 7. Anti-CD45 staining with different immunostaining techniques and microscopy techniques: immunogold-silver staining (**A–C**), alkaline phosphatase-based Vector Red staining (**D–F**) and Texas Red immunofluorescence (**G–I**). Immunogold-silver enhancement is rich in contrast to nuclear Fast Red staining in bright-field microscopy (**A**) and creates a complete segmentation in epipolarization imaging (**B** and **C**, pseudocolor depiction). Bright-field image of Vector Red immunostaining and methyl green counterstaining is displayed in **D**. Gray-scale imaging using the custom-designed absorbance filter and image inversion (**E**) shows segmentation of the Vector Red immunostaining with minimal background staining (**F**, pseudocolor depiction). Texas Red immunofluorescence is shown as two-channel depiction with DAPI counterstaining (**G**). Note that autofluorescence of lung tissue is obvious (**H**, fluorescence gray-scale image; **I**, pseudocolor depiction).

time and therefore suitability of DAB for densitometric quantitation was demonstrated in all studies.^{32,34,43} DAB deposition can also be quantified using reflection contrast microscopy,^{26,29} an epi-illumination technique that detects the reflected light from metal particles.²⁴ DAB is, however, a highly toxic substance and has thus to be handled with particular care and requires special disposal; it was therefore not considered to be appropriate for routine diagnostic immunostaining.

Antibody-linked immunogold particles can be clearly visualized light microscopically after amplification by silver enhancement and use of epipolarization microscopy technique.^{16,20,21,44} The combination of immunogold-silver staining and epipolarization microscopy is a highly sensitive method applicable for both immunohistochemistry and *in situ* hybridization, producing excellent segmentation of the bright-appearing immunopositive signal as shown in the current and previous studies.^{22,23,45,46}

Image analysis of our standard blocks, which resemble tissue samples with a defined concentration of label, demonstrated a linear relationship between epipolarization intensity and antibody concentration, as was anticipated from a on-slide enzyme-linked immunosorbent assay technique combined with epipolarization microscopy.²⁰ In addition, unrestricted light stability throughout time and a spatially precise signal with no out-of-focus signal recommends the immunogold-silver technique for quantitative studies with evaluation of staining intensity. Measurement of CD45 immunogold-staining intensity of the lung tissue with epipolarization technique showed the lowest scattering of data of all methods presently investigated. High sensitivity, excellent segmentation, sharp contrast in the bright-field in combination with nuclear counterstains like nuclear Fast Red or methyl green, and the possibility of permanent mounting and long-term storage are further advantages of this technique. However, in cryosections immunogold-silver staining produces more background than in paraffin sections, which limits the applicability for quantitative evaluation. Moreover, when assessing pathology samples, the frequent occurrence of foreign body material, such as anthracotic pigmentation in human lung tissue, represents a problem for the segmentation of the immunostaining signal even in epipolarization microscopy.

An alternative for use in paraffin- and cryosections, with no limitations concerning the appearance of foreign body material, is the alkaline phosphatase-based immunostaining with development of different colored substrates. Quantitative evaluation of alkaline phosphatase-based immunohistochemistry has been reported in a limited number of studies, using the substrate Fast Red^{29,37,38} and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.³⁶ Fast Red was recommended for use in double-labeling studies with fluorescence.⁴⁷⁻⁴⁹ Vector Red displays nearly identical features as Fast Red, including bright red color in the bright-field and intense fluorescence detectable in rhodamine or Texas Red filter systems. In the present study, linearity of Vector Red absorbance and amount of incorporated enzyme, development time, or section thickness were shown, demonstrating suitability of this tool for quantitative assessment. Control of the section thickness was performed by measurement of fluorescein-isothiocyanate autofluorescence, as previously described.^{41,50} Depending on the required accuracy of measurement a correction of section thickness may additionally be considered by applying a correction formula.¹ The section thickness may be measured either by autofluorescence intensity or immunohistochemical background staining.¹

Comparison between Vector Red absorbance and fluorescence detection revealed that the linear range for absorbance measurement comprised at least 60 minutes of development time, whereas fluorescence of Vector Red was only linear between 20 and 40 minutes of development time. Measurement of absorbance is thus less critical than fluorescence when the development time has to be adjusted for optimal immunohistochemistry. An important advantage of Vector Red is the absolute light stability in absorbance measurements. Both Vector Red and Fast Red show considerable photobleaching when

exposed to mercury light in fluorescence microscopy (this study and Speel et al) which is, however, much lower and delayed compared to commonly used fluorescent labels like Texas Red, as demonstrated in the current study.

Because of photobleaching, fluorescence measurements result in higher variability of data as compared to absorbance measurements. However, fluorescent measurements can be reliably performed when taking this fact into consideration, eg, by using an automated microscope with a light shutter to reduce the time of light exposure to a minimum.^{51,52} Sections stained with common fluorescent markers or Fast Red have to be mounted in aqueous mounting medium for fluorescence microscopy, which hinders storage and long-term stability. Vector Red-stained specimens, in contrast, can be permanently mounted with coverslips and be conserved for months or years without loss of staining intensity. Good results were obtained in the present study with the water-soluble mounting medium Crystallmount, eg, for mounting of Fast Red-stained specimens, which hardens like any other permanent mounting medium and provides good optical quality when covered with immersion oil.

The counterstaining of Vector Red staining has to be selected carefully if the slides are to be observed in the bright-field and fluorescence microscopy, because common nuclear stains used in bright-field microscopy show intense fluorescence. In fluorescence microscopy of Vector Red-stained structures, counterstaining with methyl green was found to be inappropriate, because of the extremely strong fluorescence of methyl green throughout almost the entire fluorescent spectrum. Mayer's hematoxylin, in contrast, was suitable for use in bright-field and fluorescence microscopy because segmentation of the immunostaining was possible. In contrast to Mayer's hematoxylin, other commonly used hematoxylin counterstains again display intense fluorescence. As expected, DAPI is suitable for counterstaining in fluorescence microscopy, but is unfortunately not useful for bright-field microscopy. For quantitative evaluation with absorbance measurement, we found methyl green to be the ideal counterstain, because transmission spectra of Vector Red and methyl green do not overlap and are thus easy to separate with appropriate filters. For Fast Red staining, with either bright-field or fluorescence microscopy, the choice of counterstain is limited to water-soluble dyes, and thus Mayer's hematoxylin is a suitable nuclear stain for this purpose.

Additional problems in using fluorescence microscopy for quantitative evaluation of staining intensity may arise from tissue-specific autofluorescence and out-of-focus fluorescence, which both influence accuracy of measurements. Autofluorescence is particularly disturbing in lung tissue, because of the high content of elastic fibers within the alveolar septum and in pulmonary vessels, which may hinder segmentation of the fluorescence signal. When measuring epipolarization signals of immunogold-silver deposits an interference by background signals with the immunopositive signal does not occur because silver-based immunohistochemistry is not influenced by the

presence of endogenous enzymatic activity and thus exact segmentation of the signal is possible.⁵³

It is important to mention that the described methods of quantification do not yield absolute quantitative data, but show relative changes of protein expression in different samples. Prerequisite for obtaining reliable and reproducible results are equal conditions of tissue processing, which requires standardization and calibration of each step of tissue processing. This includes parameters such as period of time until fixation starts, type of fixative and length of fixation, size of tissue samples, storage conditions, and parameters of tissue embedding. For each different antigen, which is to be localized and measured, the individual staining conditions again have to be optimized. Variations between antigens such as the amount of antigen, stability, or metabolic break down determine which technique of tissue processing may be used: paraffin-embedded tissue *versus* frozen tissue, necessity of antigen retrieval, or variable staining conditions (ie, type and concentration of antibodies, incubation periods). In experimental applications the conditions of tissue processing can more easily be held equal than in clinical applications. If certain parameters of processing clinical tissue samples cannot be fully standardized, at least the influence of these parameters on the immunostaining should be analyzed. The grade of standardization influences the accuracy of the quantitation method, which means that low standardization results in high variability of measurement and thus differences between groups have to be high to be detected. The grade of standardization, which is required for accurate measurement, in the last instance depends on the individual antigen, its expression, stability, and the differences of variation expected to be measured in different tissue samples. If sufficient standardization, calibration, and proper controls for quantitation are ensured, quantitative microscopy can be a helpful tool in experimental and diagnostic pathology.^{1,39,40}

In conclusion, microdensitometry of Vector Red absorbance is a suitable method for quantification of staining intensity and meets current requirements for a stable, reproducible, and economical technique to be applied in diagnostic pathology. Applicability in paraffin-embedded tissue as well as in cryosections, excellent segmentation, linearity over a wide range, light stability, and feasibility for permanent mounting and for long-term storage are the outstanding features of this approach. Absorbance measurement does not require expensive equipment and can be performed with a common light microscope, an appropriate absorbance filter, and a stabilized transformer for steady illumination. In comparison with other staining and measurement techniques reported in the literature and those presently investigated in parallel, we consider microdensitometry of Vector Red absorbance as being the most suitable approach for standardized quantitative evaluation.

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References

1. Fritz P, Wu X, Tuzcek H, Mulhaupt H, Schwarzmann P: Quantitation in immunohistochemistry. A research method or a diagnostic tool in surgical pathology? *Pathologica* 1995, 87:300–309
2. Mariuzzi GM, Collan YUI: Some reflections on the history, and presence of quantitative pathology. *Pathologica* 1995, 87:215–220
3. Hall TL, Fu YS: Biology of disease. Applications of quantitative microscopy in tumor pathology. *Lab Invest* 1985, 53:5–21
4. Stoward PJ, Nakae Y, Van Noorden CJF: The everchanging advances in enzyme histochemistry, 1986–1996. *Eur J Histochem* 1998, 42:35–40
5. Van Noorden CJF, Jonges GN: Analysis of enzyme reactions in situ. *Histochem J* 1995, 27:101–118
6. Mariuzzi GM, Tosi P: Quantitative microscopy today. *Appl Pathol* 1986, 4:5–8
7. Fritz P, Tuzcek HV, Böffinger B, Schwarzmann P, Schieszl S, Wu X, Kleine B, Blödorn J, Mulhaupt H: Immunohistochemical quantification of steroid receptors and other prognosis factors in human breast cancer patients. *Prog Histochem Cytochem* 1992, 26:146–158
8. Bacus SS, Goldschmidt R, Chin D, Moran D, Weinberg D, Bacus JW: Biological grading of breast cancer using antibodies to proliferating cells and other markers. *Am J Pathol* 1989, 135:783–792
9. Bacus S, Flowers JL, Press MF, Bacus JW, McCarty KS: The evaluation of estrogen receptor in primary breast carcinoma by computer-assisted image analysis. *Am J Clin Pathol* 1988, 90:233–239
10. Zhou R, Hammond EH, Sause WT, Rubin P, Emami B, Pilepich MV, Asbell SD, Parker DL: Quantitation of prostate-specific acid phosphatase in prostate cancer: reproducibility and correlation with subjective grade. *Mod Pathol* 1994, 7:440–448
11. Pauletti G, Godolphin W, Press MF, Slamon DS: Detection and quantitation of HER-2/neu gene amplification in human breast cancer archival material using fluorescence in situ hybridization. *Oncogene* 1996, 13:53–72
12. Ross JS, Fletcher JA: HER-2/neu (c-erbB-2) gene and protein in breast cancer. *Am J Clin Pathol* 1999, 112:S53–S67
13. Pandha HS, Martin LA, Rigg A, Hurst HC, Stamp GWH, Sikora K, Lemoine NR: Genetic prodrug activation therapy for breast cancer: a phase I clinical trial of erbB-2-directed suicide gene expression. *J Clin Oncol* 1999, 17:2180–2189
14. Haajman JJ: Immunofluorescence: quantitative considerations. *Acta Histochem* 1988, 35:S77–S83
15. Polak JM, VanNoorden S (Eds): *Immunocytochemistry. Modern Methods and Applications*. Bristol, John Wright and Sons Ltd., 1986
16. Pluta M (Ed): *Advanced Light Microscopy. Measuring Techniques*. Amsterdam, Elsevier Science Publishers B. V., 1993
17. Hansen GH, Wetterberg LL, Sjöström H, Noren O: Immunogold labelling is a quantitative method as demonstrated by studies on aminopeptidase N in microvillar membrane vesicles. *Histochem J* 1992, 24:132–136
18. Slater M, Mason RS: The determination of comparable labeling densities in quantitative immunoelectron microscopic double labeling studies. *Biotech Histochem* 1994, 69:127–135
19. Lucocq J: Quantitation of gold labelling and antigens in immunolabelled ultrathin sections. *J Anat* 1994, 184:1–13
20. Gao K, Morris RE, Wie C: Epipolarization microscopic immunogold assay: a combination of immunogold silver staining, enzyme-linked-immunosorbent assay and epipolarization microscopy. *Biotech Histochem* 1995, 70:211–216
21. Gao K, Cardell EL: Pseudocolor image processing of PEPCCK subcellular distribution in rat hepatocytes shown with IGSS and epipolarization microscopy. *J Histochem Cytochem* 1994, 42:1651–1653
22. Ermer L, Ermer M, Goppelt-Strube M, Walrath D, Grimminger F, Steudel W, Ghofrani HA, Homberger C, Duncker HR, Seeger W: Cyclooxygenase isoenzyme localization and mRNA expression in rat lungs. *Am J Respir Cell Mol Biol* 1998, 18:479–488
23. Ermer L, Ermer M, Goppelt-Strube M, Merkle M, Duncker HR, Grimminger F, Seeger W: Rat pulmonary cyclooxygenase-2 expression in response to endotoxin challenge: differential regulation in the various types of cells in the lung. *Am J Pathol* 2000, 156:1275–1287
24. Landegent JE, Jansen indeWal N, Ploem JS, van der Ploeg M: Sensitive detection of hybridocytochemical results by means of reflection-contrast microscopy. *J Histochem Cytochem* 1985, 33:1241–1246

25. Cornelese-ten Velde I, Wiegant J, Tanke HJ, Ploem JS: Improved detection and quantification of the (immuno) peroxidase product using reflection contrast microscopy. *Histochemistry* 1989, 92:153–160
26. Cornelese-ten Velde I, Bonnet J, Tanke HJ, Ploem JS: Reflection contrast microscopy. Visualization of (peroxidase-generated) diaminobenzidine polymer products and its underlying optical phenomena. *Histochemistry* 1988, 89:141–150
27. Prins FA, vanDeimen-Steenvoorde R, Bonnet J, Cornelese-ten Velde I: Reflection contrast microscopy of ultrathin sections in immunocytochemical localization studies: a versatile technique bridging electron microscopy with light microscopy. *Histochemistry* 1993, 99:417–425
28. Chieco P, Jonker A, Melchiorri C, Vanni G, vanNoorden CJF: A user's guide for avoiding errors in absorbance image cytometry: a review with original experimental observations. *Histochem J* 1994, 26:1–19
29. Zhou R, Hammond EH, Parker DL: A multiple wavelength algorithm in color image analysis and its applications in stain decomposition in microscopy images. *Med Phys* 1996, 23:1977–1986
30. Zhou R, Hammond EH, Parker DL: Quantitative peroxidase-antiperoxidase complex-substrate mass determination in tissue sections by a dual wavelength method. *Anal Quant Cytol Histol* 1992, 14:73–80
31. Rahier J, Stevens M, DeMenten Y, Henquin JC: Determination of antigen concentration in tissue section by immunodensitometry. *Lab Invest* 1989, 61:357–363
32. Pool CW, Madlener S, Diegenbach PC, Sluiter AA, van der Sluis P: Quantification of antiserum reactivity in immunocytochemistry. *J Histochem Cytochem* 1984, 32:921–928
33. Nibbering PH, Leijh PCJ, vanFurth R: A cytophotometric method to quantitate the binding of monoclonal antibodies to individual cells. *J Histochem Cytochem* 1985, 33:453–459
34. Streefkerk JG, vanderPloeg M, vanDuijn P: Agarose beads as matrices for proteins in cytophotometric investigations of immunohistoperoxidase procedures. *J Histochem Cytochem* 1975, 23:243–250
35. Mize RR, Holdefer RN, Nabors LB: Quantitative immunocytochemistry using an image analyzer. I. Hardware evaluation, image processing, and data analysis. *J Neurosci Methods* 1988, 26:1–24
36. Nibbering PH, Marijnen JGJ, Raap AK, Leijh PCJ, vanFurth R: Quantitative study of enzyme immunocytochemical reactions performed with enzyme conjugates immobilized on nitrocellulose. *Histochemistry* 1986, 84:538–543
37. van der Loos CM, Marijanowski MMH, Becker AE: Quantification in immunohistochemistry: the measurement of the ratios of collagen types I and II. *Histochem J* 1994, 26:347–354
38. van Duijn P, Pascoe E, vanderPloeg M: Theoretical and experimental aspects of enzyme determination in a cytochemical model system of polyacrylamide films containing alkaline phosphatase. *J Histochem Cytochem* 1967, 15:631–645
39. Fritz P, Hönes J, Lutz D, Mulhaupt H, Mischlinski A, Dörner A, Schwarzmann P, Tuzcek HV, Müller W: Quantitative immunohistochemistry: standardization and possible application in research and surgical pathology. *Acta Histochem* 1989, 37:S213–S219
40. Becker RL: Standardization and quality control of quantitative microscopy in pathology. *J Cell Biol* 1993, 17:199–204
41. van de Lest CHA, Versteeg EMM, Veerkamp JH, vanKuppevelt TH: Elimination of autofluorescence in immunofluorescence microscopy with digital image processing. *J Histochem Cytochem* 1995, 43:727–730
42. Geerts A, Roels F: Quantitation of catalase activity by microspectrophotometry after diaminobenzidine staining. *Histochemistry* 1981, 72:357–367
43. Nabors LB, Songu-Mize E, Mize RR: Quantitative immunocytochemistry using an image analyzer. II. Concentration standards for transmitter immunocytochemistry. *J Neurosci Methods* 1988, 26:25–34
44. de Waele M, Renmans W, Segers S, Jochmans K, vanCamp B: Sensitive detection of immunogold-silver staining with darkfield and epi-polarization microscopy. *J Histochem Cytochem* 1988, 36:679–683
45. Gao K, Morris RE, Giffin BF, Cardell RR: Immunogold-silver staining and epipolarized light microscopic detection of phosphoenolpyruvate carboxykinase and glycogen phosphorylase in rat liver. *Histochemistry* 1993, 99:341–346
46. Shires M, Goode NP, Crellin DM, Davison AM: Immunogold-silver staining of mesangial antigen in Lowicryl K4M- and LR Gold-embedded renal tissue using epipolarization microscopy. *J Histochem Cytochem* 1990, 38:287–289
47. Murdoch A, Jenkinson EJ, Johnson GD, Owen JJT: Alkaline phosphatase-Fast Red, a new fluorescent label. Application in double labelling for cell surface antigen and cell cycle analysis. *J Immunol Methods* 1990, 132:45–49
48. Speel EJM, Schutte B, Wiegant J, Ramaekers FCS, Hopman AHN: A novel fluorescence detection method for in situ hybridization, based on the alkaline phosphatase-Fast Red reaction. *J Histochem Cytochem* 1992, 40:1299–1308
49. van der Loos CM, Becker AE: Double epi-illumination microscopy with separate visualization of two antigens: a combination of epipolarization for immunogold-silver staining and epi-fluorescence for alkaline phosphatase staining. *J Histochem Cytochem* 1994, 42:289–295
50. Brismar H, Patwardhan A, Jaremko G, Nyengaard J: Thickness estimation of fluorescent sections using a CSLM. *J Microsc* 1996, 184:106–116
51. Pluta M (Ed): *Advanced Light Microscopy. Specialized Method*. Amsterdam, Elsevier Science Publishers B. V., 1989
52. DiGuiseppi J, Inman R, Ishihara A, Jacobson K, Herman B: Applications of digitized fluorescence microscopy to problems in cell biology. *BioTechniques* 1985, 3:394–403
53. Scopsi L: *Silver-Enhanced Colloidal Gold Method*. Edited by MA Hayat. San Diego, Academic Press Inc., 1989, pp 252–288