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Journal of Pharmaceutical and Biomedical Analysis
18 (1998) 555–564

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Chemiluminescence imaging in bioanalysis

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Received 15 May 1998; received in revised form 3 August 1998; accepted 15 September 1998

Abstract

The development, analytical performance and applications of chemiluminescence imaging as a tool for quantitative analyte localization in target biological specimens are described. The detection of acetylcholinesterase activity both in array format and on a target surface are described. A proposed application of the method is a 384 well microtiter format assay for high throughput screening of acetylcholinesterase inhibitors such as tacrine, a drug widely used in the treatment of Alzheimer's disease, and two recently developed analogues. The chemiluminescent system in conjunction with optical microscopy allowed localization of acetylcholinesterase in brain tissue sections. We also describe the chemiluminescent immunohistochemical localization of interleukin 8 in *Helicobacter pylori* infected gastric mucosa cryosections and an in situ hybridization assay for the detection of herpes simplex virus DNA in single cells. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Biospecific probes; Chemiluminescence imaging; Chemiluminescence coupled enzymatic reactions; Chemiluminescence immunohistochemistry; Chemiluminescence in situ hybridization; High throughput screening

1. Introduction

Direct detection of a few molecules in a complex biological matrix is a challenge for analytical chemistry and, when successful, is a powerful tool in clinical, pharmaceutical chemistry and molecular biology. Chemiluminescence (CL) has been widely used for this purpose in various analytical techniques and, in more recent years, many CL ultrasensitive assays have been developed that use enzyme labels in immuno- or gene probe assays, or that utilize coupled enzymatic reactions, all of which terminate in emission of light [1–6].

For use in these assays, highly efficient CL substrates have been developed that can detect enzymes such as alkaline phosphatase (AP) or horseradish peroxidase (HRP) at femtomole levels [7–11].

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PII: S0731-7085(98)00209-X

Chemiluminescence offers the unique advantage that light is emitted by a specific reaction, thus avoiding the background emission typical of fluorescence; in addition, the phenomenon occurs in a time frame of a few seconds, thus rendering CL techniques very rapid.

Light can be measured efficiently with cooled photomultiplier tube (PMT) and, more recently, can also be imaged on a target surface with highly sensitive cooled back-illuminated charge-coupled device (CCD). In particular, a biospecific CL reaction can be directly imaged on a given surface such as gel, blot, microtiter plate or, in conjunction with an optical microscope, tissue sections, single cells and microchip device [12,13].

CL imaging systems are now currently used in Western or Southern blot and related techniques, offering the advantages of permitting semiquantitative analysis while avoiding the use of radioisotopes and long exposure times with a photographic film [14]. The main analytical challenge for CL imaging is to combine the high resolution achievable for the visible light emitted by the CL probe, which is in the order of 500–800 nm, and the high standard achieved in microfabrication to develop microarray device.

In previous papers we demonstrated the usefulness of CL imaging in immunohistochemical and in situ hybridization reactions and the possibility of performing quantitative analysis on a target surface of a given CL probe such antibodies or cDNAs [15–18].

Different probes can be also imaged in the same sample, permitting the simultaneous detection of more than one analyte [19]; with the use of CL in conjunction with optical microscopy it is possible to image reactions in a very small vessel such as a microchip with a reaction chamber of few μl, under which condition a very diluted sample can be analyzed. The detection limit is related to the quantum yield of the CL reaction used and to the sensitivity of the CCD camera used, and it is usually found to be in the order of 10^{-16}–10^{-19} mol.

A microscope can be attached to the imaging apparatus and the reaction kinetics continuously monitored with a CCD camera. With this kind of set-up, many samples can be simultaneously analyzed by immobilizing biospecific probes in different areas of an array format.

At the macro-scale level CL imaging offers the possibility of imaging microtiter plates consisting of 96 or 384 wells, thus allowing high throughput screening (HTS) assays for drugs to be developed by automating all the preanalytical dispensing steps.

In this paper we describe the development and analytical performance of CL imaging for the detection of acetylcholinesterase (AChE) activity both in array format and in brain tissue sections.

The CL detection of this enzyme was based on coupled enzymatic reactions involving choline oxidase and horseradish peroxidase as the indicator enzymes leading to light emission and using luminol/enhancer to detect the H_2O_2 formed. This system was also optimized for imaging purposes, which involved the exact determination of the overall enzymatic kinetics as well as diffusion of the substrates and indicator enzymes in order to determine the quality of the imaged CL signal.

A proposed application of the method is a 384 well microtiter format assay for HTS of AChE inhibitors such as tacrine, a drug widely used in the treatment of Alzheimer's disease, and two recently synthesized analogues recognized to be more selective and less hepatotoxic.

The CL system coupled with optical microscopy allowed localization of this enzyme in brain tissue sections.

We also describe the CL immunohistochemical localization of interleukin 8 (IL 8) in *Helicobacter pylori* infected gastric mucosa cryosections and an in situ hybridization assay for the detection of herpes simplex virus (HSV) DNA in single cells.

2. Experimental

2.1. Chemicals

Peroxidase (type VI-A, from horseradish, 1100 U mg^{-1}), acetylcholinesterase (type V-S, from electric eel, 1000–2000 U mg^{-1}), choline oxidase (from *Alcaligenes* genus approximately 10 U mg^{-1}), 5-amino-2,3-dihydro-1,4-phthalazinedone (luminol sodium salt), and AP-labeled goat anti-

mouse antibody were purchased from Sigma, MO. The reagent 4-iodophenol was purchased from Aldrich (Milwaukee, WI). Biotinylated HSV DNA probe was purchased from Enzo Biochem (New York, NY), and streptavidin-HRP complex, from NEN Life Science Products (Boston, MA). Monoclonal mouse anti-IL 8 was kindly gifted by Dr I.J.D. Lindley (Novartis Research Institute, Vienna). For HRP detection we used ECL enhanced chemiluminescent luminol reagent (luminol/enhancer/H_2O_2) (Amersham, Amersham, UK) and SuperSignal Ultra (luminol/enhancer/stable peroxidase) (Pierce, Rockford, IL) following the manufacturers' instructions. For AP detection we used Lumi-Phos Plus (Lumigen, Southfield, MI) and CDP-Star (Tropix, Bedford, MA) following the manufacturers' instructions. All other chemicals were of high-quality analytical grade.

2.2. Imaging devices

The detection and analysis of signals were performed using a high-performance, low-light-level imaging apparatus, (Luminograph LB 980, EG&G Berthold, Bad Wilbad, Germany), which permits emitted light measurement at the single-photon level. The video system consists of a 1″ Saticon, high dynamic range pick-up tube (which is a Vidicon-type tube with Se-As-Tl light target photoconductor) linked to an image intensifier, by high transmission lenses, and also to a videoamplifier. This system is connected to a PC for quantitative image analysis, and a sample dark box is provided to prevent contact with external light. The videocamera can be connected to a Model BH-2 Optical Microscope (Olympus Optical, Tokio, Japan) also enclosed in a dark box. The system operates in the following consecutive steps: (i) samples are recorded as transmitted light; (ii) the luminescent signal is measured with an optimized photon accumulation lasting 1 min, with 2 s interval integration; and (iii) after a computer elaboration of the luminescent signal with pseudo-colors corresponding to the light intensity, an overlay of the images on the screen provided by the transmitted light and by the luminescent signal allows the spatial distribution of the target analytes to be localized and evaluated. The light emission from each cell is quantified by defining a fixed area and counting the number of photon fluxes from within this area.

Alternatively, when higher detectability was required, a Luminograph LB 981 (EG&G Berthold, Bad Wilbad, Germany) based on a back-illuminated cooled CCD was used. The instrument set up and CL imaging processing is quite similar to the LB 980 luminograph.

2.3. AChE detection in solution and AChE inhibitors assay

A CL reagent solution containing 0.1 mM acetylcholine, 0.3 U ml^{-1} choline oxidase, 6 mM luminol, 2 mM 4-iodophenol, 0.22 U ml^{-1} HRP in potassium phosphate buffer (pH 8.0; 0.5 M) was optimized for the analysis of AChE activities ranging from 10^{-2} to 10^{-5} U $well^{-1}$. A chemiluminescent cocktail containing the reagent solution and 10^{-2} U $well^{-1}$ AChE was used for the inhibition study, which is based on the principle that the AChE-dependent light emission is reduced by addition of enzyme inhibitors. Aqueous solutions of tacrine chlorohydrate and two chlorohydrate analogues (1 and 2) ranging from 1 to 100 nM were examined. The synthesis and activity of the two analogues have been reported elsewhere [20]. When 384 well polystyrene microtiter plates (Corning Costar, Acton, MA) were used, 1–2 μl of inhibitor solutions were added to 10 μl of the CL cocktail and the light emission kinetics were monitored for at least 30 min, with light accumulations lasting 1 min each.

2.4. AChE localization in rat brain

Coronal brain slices were prepared from adult Sprague–Dawley rats following a method which preserves neuronal functions [21]. Briefly, rats were anesthetized and decapitated; brains were quickly removed and immersed in cold (4°C) low-sodium, high-sucrose solution (in mM: 212.7 sucrose, 2.6 KCl, 1.23 NaH_2PO_4, 26 $NaHCO_3$, 2 $MgSO_4$, 2 $CaCl_2$ and 10 dextrose) bubbled with a mixture of 95% O_2 and 5% CO_2 at pH 7.4. Coronal slices, 100–200 μm thick, were cut using

an oscillating tissue slicer (Frederick Haer Corp., Brunswick, ME) at 4°C, collected on pre-cooled microscope glass slides and frozen at −80°C. One set of alternate sections was neutral red counterstained in order to clearly identify the cerebral structures. The remaining sections were used for the CL analysis: to each section, 40 μl of the previously described CL reagent solution were added, and light signal was acquired for 1 min using the videocamera-optical microscope device. The method for AChE localization on a target surface had been previously developed and optimized using a model system in which the enzyme was chemically immobilized on oxirane acrylic beads (250 μm diameter macroporous particles) and detected with two auxiliary enzymes in solution [13].

2.5. Immunohistochemical localization of interleukin 8 in gastric mucosa

Antral gastric mucosa biopsy specimens were taken from gastroduodenal disease patients and frozen in isopentane cooled over liquid nitrogen; 5 μm thin sections were cut in a cryostat microtome, collected onto microscope glass slides and fixed in acetone for 5 min. Sections were incubated with monoclonal mouse anti-IL 8 for 1 h at room temperature in a humidified chamber. They were washed in Tris buffered saline (TBS) for 30 min with stirring, then incubated with AP-labeled goat anti-mouse antibody for 1 h at room temperature in a humidified chamber and washed again in TBS for 30 min with stirring [22]. Sections were incubated for 15–20 min in the dark at room temperature with 20–40 μl of the CL substrate for AP, then the CL signal was acquired for 1 min.

2.6. In situ hybridization of herpes simplex virus DNA in single fibroblasts

In situ hybridization was performed as previously described [23]. Briefly, human fibroblasts infected with clinical samples known to contain HSV were fixed at 48 h post-infection in 4% paraformaldehyde, then treated with pronase and dehydrated by ethanol washes. Dehydrated cells were overlaid with 20 μl of the hybridization mixture (50% deionized formamide, 10% dextran sulfate, 250 mg ml^{-1} of calf thymus DNA and 24 ng ml^{-1} of biotin-labeled HSV DNA probe in 2X SSC buffer). Cell samples and the hybridization mixture containing the biotin-labeled probe were denaturated together by heating in a 92 ± 2°C water bath for 6 min, and then hybridized at 37°C for 3 h. After hybridization, cells were washed three times at stringent conditions. Hybridized probes were detected using streptavidin-HRP complex and the chemiluminescent substrate for HRP, with an optimized accumulation lasting 1 min.

3. Results and discussion

The detection of AChE in microtiter format was optimized to permit the simultaneous analysis of the 384 wells of the microtiter plates with a luminograph device which uses a cooled ultrasensitive CCD camera. The light emission reached a steady-state after 10–15 min, was stable for at least 10 min and its intensity was proportional to the AChE activity. The dose-response curve was linear in a range from 10^{-2} to 10^{-5} U $well^{-1}$ of AChE, with a detection limit of 10^{-5} U $well^{-1}$.

The addition of AChE inhibitors to the CL cocktail resulted in a concentration-dependent reduction in light output (Fig. 1). Fig. 2 shows a 384 well microtiter plate in which the activity of AChE inhibitors at different concentrations was evaluated. After the initial light suppression, the photon emission resumed starting from the lower concentrations and reached values similar to those of the control wells (panels 1, 2). Then a light output decrease was observed in all wells, which is consistent with the CL reaction kinetics (panels 3, 4), suggesting that the inhibition activity has to be evaluated during the steady-state emission. With the luminograph software it was possible to calculate the integral of the light emitted by each well or to directly report the dose-dependent inhibition effect of the drugs. The method fulfilled all the requirements of precision since the coefficient of variation calculated over different sets of experiments never exceeded 4%. Results obtained with conventional color producing AChE substrate

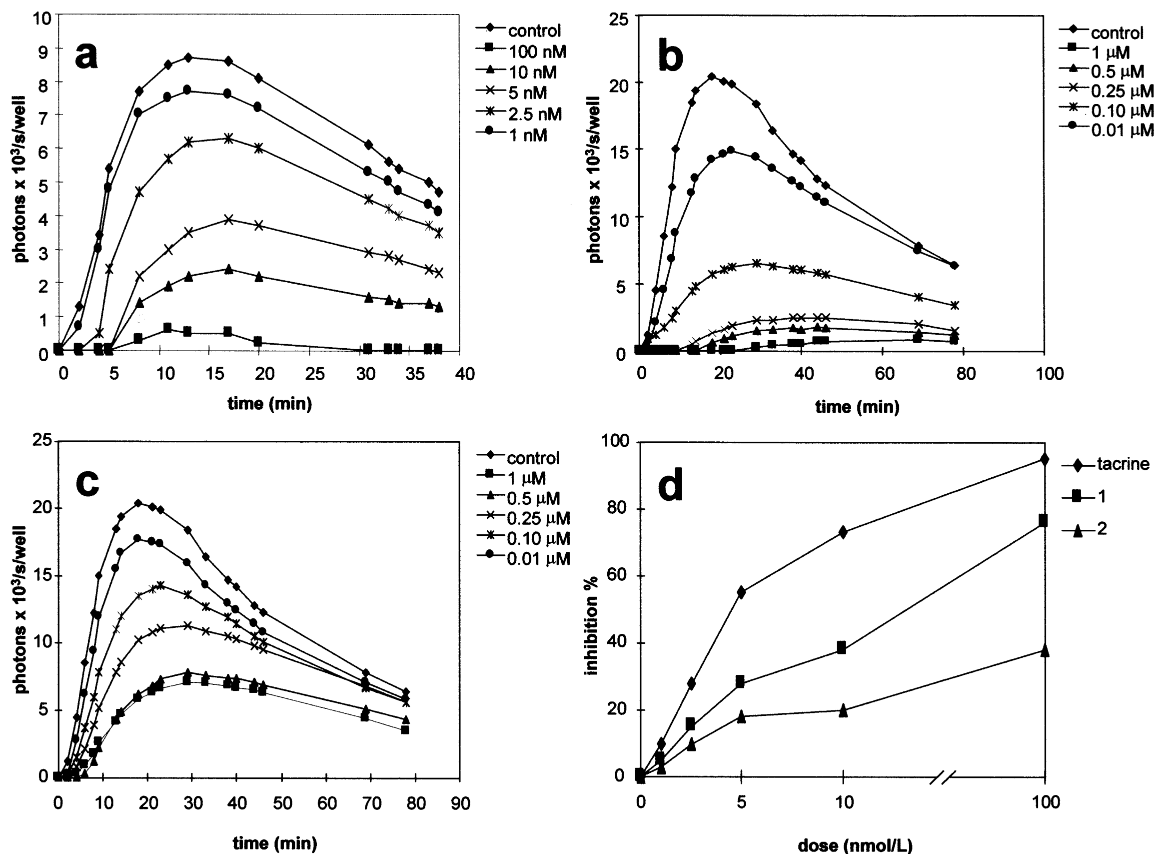

Fig. 1. Kinetic profiles of the light output deriving from AChE catalyzed chemiluminescent reaction in the presence of different concentrations of tacrine (a), analogue 1 (b) and analogue 2 (c). Comparison of the inhibition effects of the three drugs as a function of concentration (d).

[20,24] are in agreement with those achieved with CL detection, demonstrating the good accuracy of this method which, in addition, had a detection limit 3 decades lower than that of colorimetric analysis. When compounds poorly soluble in water have to be analyzed, a mixed solvent containing not more than 10-20% of dimethyl sulfoxide (DMSO) or dimethylformamide (DMF) should be used, and the drug inhibition effect has to be reported considering the quenching of light emission due to DMSO or DMF itself.

As far as analyte localization and quantification on a target surface is concerned, the biological specimen must be prepared to allow access of the substrate to the enzyme active sites. The best arrangements for achieving good resolution is to use a direct CL substrate for the enzyme, as in the case of dioxetane phosphate for alkaline phosphatase or luminol/enhancer/H_2O_2 for horseradish peroxidase. Coupled enzymatic reactions terminating with light emission are widely used to detect many enzymes or their substrates in solution [25–27] but have often presented limits when used for imaging.

The developed CL reagent solution for AChE proved to be suitable for analyte localization on a target surface: it allowed direct localization of the spatial distribution of the enzyme in rat brain sections, with a sharp CL signal localized in cholinergic neurons but very low background emission (Fig. 3). The relative concentrations of the components of the CL reagent solution were

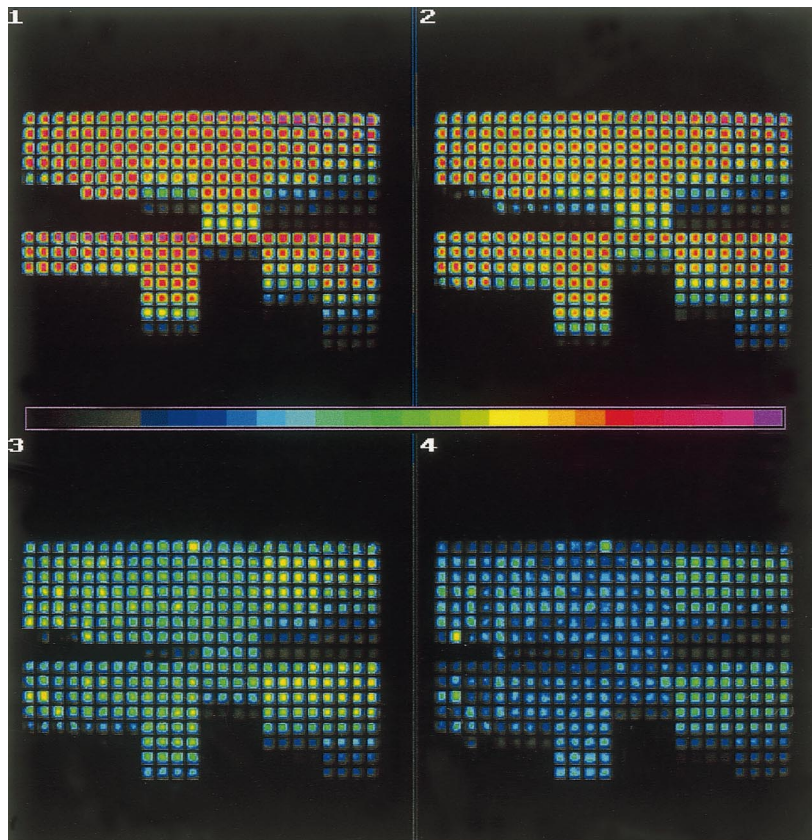

Fig. 2

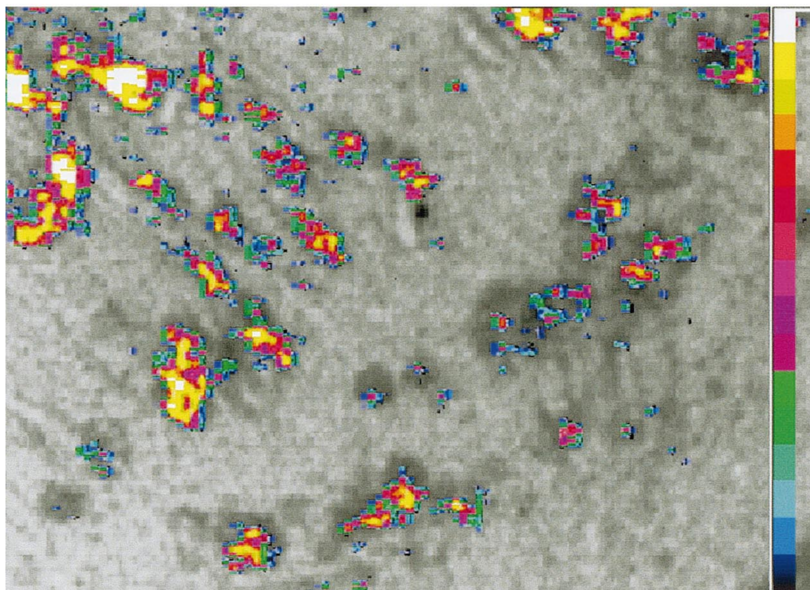

Fig. 3

Fig. 2. Example of chemiluminescence imaging of 384 well microtiter plate for high throughput screening assay of AChE inhibitors. The chemiluminescent signal is recorded at different times intervals for kinetic and dose-dependence studies.

Fig. 3. Chemiluminescence imaging of AChE in coronal rat brain slice (100 μm thick), with the light signal sharply localized in cholinergic neurons. Pseudocolor ruler on the right shows the relative light intensity.

optimized in order to avoid the diffusion of the light emission species in the surrounding solution.

The analyte concentration in the tissue sample could be quantified using a calibration curve obtained by immobilizing different amounts of AChE on a target surface such as oxirane acrylic beads, as reported in a previous paper [13]. The standard curve ($y = 13.732x + 0.723$, $R^2 = 0.9921$) was linear in a range 0.1–1.0 mU bead^{-1}, with a detection limit of 0.1 mU bead^{-1}.

This imaging system could be a useful tool to study both the patho-physiological role of AChE distribution in brain and the effect of in vivo administration of enzyme inhibitor drugs, providing a system which is more predictive than in vitro assay of inhibition activity.

Chemiluminescence immunohistochemical technique was able to localize and quantify IL 8 in the gastric mucosa. A CL signal for IL 8 was observed in gastric epithelium in all the specimens studied (Fig. 4), and increased IL 8 in epithelial cells in *H. pylori* infection was demonstrated, thus confirming earlier results achieved with immunofluorescence technique [28]. Computer processing of the IL 8 image allows direct visualization of the variations in chemokine distribution within epithelial cells and mucosal tissues associated with disease processes. Focal areas of high IL 8 immunoreactivity can be visualized in epithelial cells, thus permitting the potential analysis of host epithelial mediator changes in relation to infection with pathogenic agents such as *H. pylori*.

Serial sections of each sample were examined. With a well standardized section thickness achieved using appropriate cryostat, the intensities of the light emission deriving from the same defined areas in consecutively cut sections were quite similar, with a coefficient of variation of 5–6%. The CL imaging assay proved to be reproducible, thus suitable for quantitative analysis. Tissue section thickness is a critical point when quantitative data are required, since increasing the thickness of slices results in a linear increase in detected photon intensity [29].

The main advantages of using immunochemiluminescence over conventional colorimetric technique are that it is more sensitive and that it allows quantification of tissue antigens. When compared with fluorometric detection, chemiluminescence shows an improved signal to noise ratio, thus higher specific detectability; furthermore, it provides more precise and accurate quantitative results. Moreover, relative concentration or activity of an analyte in a given specimen can be obtained more easily, permitting to determine quantitative topographical distribution.

Immunochemiluminescence technique could be a useful tool to investigate host-pathogen interactions, with particular respect to inflammatory mediators in epithelial cells such as IL 8 which is upregulated in response to infectious agents.

In the chemiluminescence in situ hybridization assay, positive signals for the presence of HSV DNA were observed in infected cells fixed at 48 h post-infection with sharp topographical localization, no cross-reactions, low background and good preservation of cellular morphology (Fig. 5). Indeed, a series of control experiments proved irrefutably that the chemiluminescent in situ hybridization reaction was detecting HSV nucleic acid sequences specifically: (i) no specific signal was detected when uninfected cells were treated with labeled probes; (ii) no specific signal was noted when cytomegalovirus or Epstein Barr virus infected cells were hybridized with the HSV labeled probe; and (iii) no specific signal was detected when HSV infected cells were treated with the unlabeled probe and with streptavidin-HRP complex. When chemiluminescence and colorimetric in situ hybridization assays were compared, after processing the samples in the same run and with the same batch of probe, the two methods showed a high correlation and, in addition, a higher number of positive cells was detected using the CL method, the difference being statistically significant.

4. Conclusions

Chemiluminescence imaging has proven to be a suitable tool for the development of both high throughput screening assays of drugs, and enzymatic, immunohistochemical and in situ hybridization methods for analyte localization on a target surface, when the system is coupled with optical microscopy.

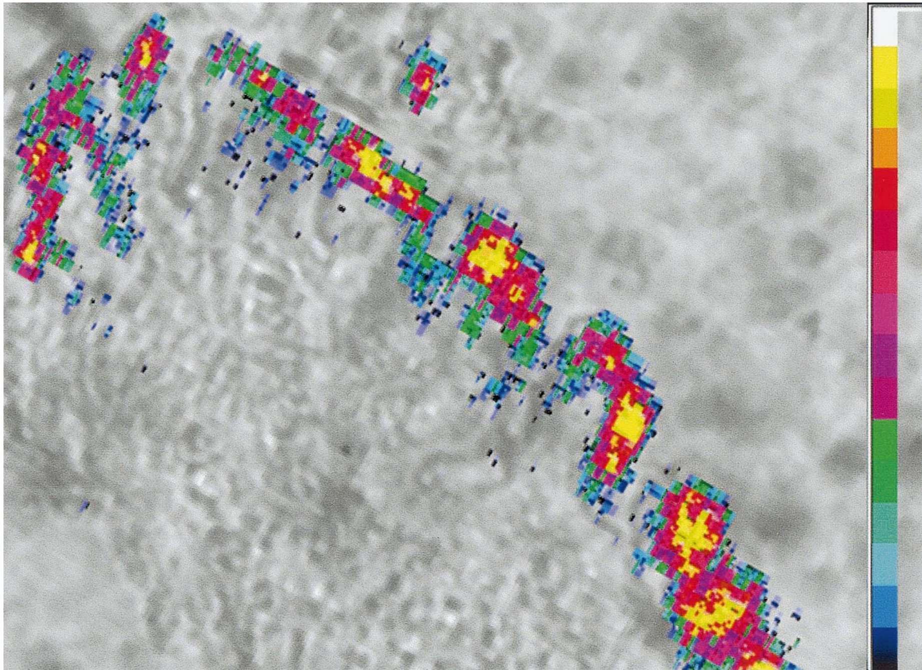

Fig. 4

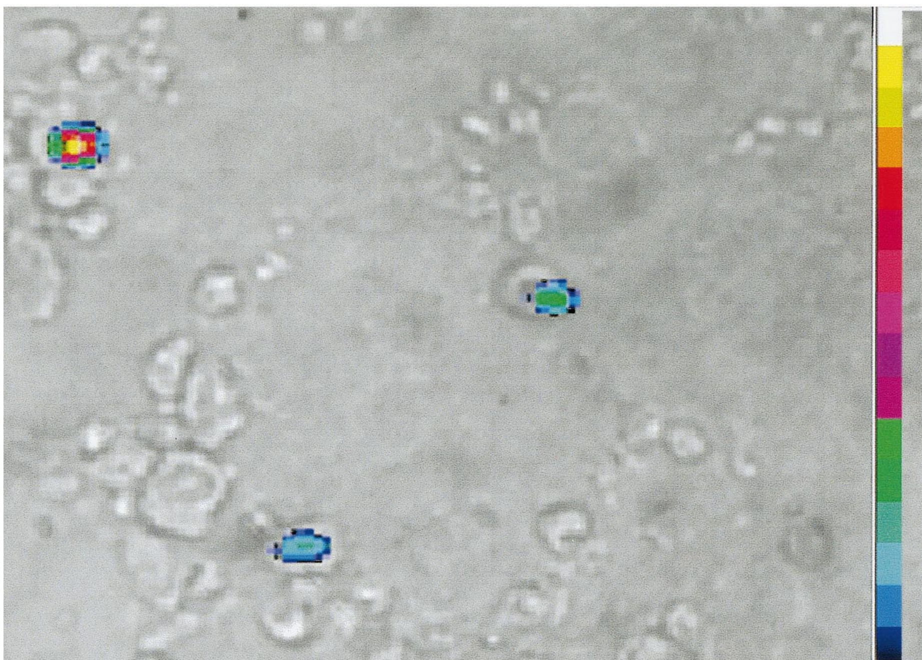

Fig. 5

Fig. 4. Chemiluminescence immunohistochemical localization of IL 8 in *H. pylori* infected gastric mucosa section, showing high signal and focal distribution in epithelial cells. Pseudocolor ruler on the right shows the relative light intensity.
Fig. 5. Chemiluminescence in situ hybridization revealing the presence of HSV DNA in infected fibroblasts. Pseudocolor ruler on the right shows the relative light intensity.

Using a CCD camera, 384 well black polystyrene microtiter plates and CL reagents that quickly (a few minutes) reach a steady-state of light output, an automated system can be easily set up to screen more than 5000 samples h^{-1}. The high detectability of CL labels makes the technique very sensitive, thus allowing analysis of small sample amounts and very diluted specimens.

Basically, for chemiluminescence imaging to be used in place of conventional radioactive or colorimetric detection techniques for localizing and quantifying biospecific probes such as enzymes, antibodies and cDNAs, two requirements must be met. First, the reaction must occur with glow-type light kinetics: this permits easy handling and standardization of the experimental conditions; moreover, it is necessary for quantitation of the labeled probe since the steady-state light intensity (photons $s^{-1}pixel^{-1}$) is directly related to the enzyme activity. The second requirement is that the chemistry and kinetics of the process must be such that light emission is limited to the area where the probe is localized.

The overall results have demonstrated that chemiluminescence detection can be successfully used for such techniques. The chemiluminescent detection of AChE and AP- or HRP-labeled probes was superior to colorimetric technique. Once standardized, CL imaging is suitable for quantitative detection of analytes, which represents another important advantage over conventional fluorometric methods. In addition, chemiluminescence uses relatively simple instrumentation that avoids the use of radioactive probes to achieve similar performance.

CL detection can be applied to various types of samples such as single cells, frozen and paraffin-embedded tissue sections. Since unstained samples are examined, the morphological structure is often not well defined. Thus, a further improvement in CL technique would be the enhancement of the transmitted light image quality by staining the samples with proper dyes either before or after CL measurement, provided that the staining does not interfere with the CL reaction. Moreover, simultaneous detection of two or more CL probes in the same sample is possible [19], thus improving the diagnostic significance of such technique.

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