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Invited Review

Chemiluminescence: a sensitive detection system in in situ hybridization

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Summary. Chemiluminescence is the light emission produced by a chemical reaction in which chemically excited molecules decay to the ground state. The phenomenon is utilized in various analytical techniques in which small amounts of analytes or enzymes can be detected and quantified by measurement of the light emitted by bio- or chemiluminescent reactions. Recently chemiluminescence has been proposed as a valid alternative to radioactive or colorimetric methods in in situ hybridization assays, in which target nucleic acids are localized by labeled probes inside individual cells with the preservation of cell morphology. Chemiluminescence in situ hybridization is performed using probes that are detected using enzymes with their appropriate chemiluminescent substrates. The luminescent signal from the hybrid formation is detected, analysed and measured with a high performance low light level imaging apparatus connected to an optical microscope and to a personal computer for quantitative image analysis. Generally, the instrumental system to detect positive signals after in situ hybridization operates in three steps: firstly tissue structures and cells are recorded in transmitted light then the luminescent signal is measured with an optimized photon accumulation; and then, after a computer elaboration of the luminescent signal with pseudocolors corresponding to the light intensity, an overlay of the two images on the screen provided by the transmitted light and by the luminescent signal allows the spatial distribution of the labeled probe to be localized and evaluated.

The main advantages of chemiluminescence in situ hybridization are mainly the sensitivity, the quantification of the data, the objectivity of the evaluation and the digital imaging of the results.

The chemiluminescence in situ hybridization assay, which can be applied to cell smears, archival frozen and paraffin embedded tissue samples, can be a useful tool for a sensitive and specific diagnosis of viral infections and for the detection and study of specific genic sequences inside the cells. The use of the chemiluminescent in situ hybridization assay is also promising for an estimation and quantification of nucleic acids present in tissue samples or cellular smears and for imaging gene expression in cells.

Key words: Chemiluminescence, In situ hybridization, Labeled probes, Chemiluminescent substrates

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Introduction

Chemiluminescence is the light emission produced by a chemical reaction in which chemically excited molecules decay to the ground state. The phenomenon is utilized in various analytical techniques in which small amounts of analytes or enzymes can be detected and quantified by measurement of the light emitted by bio- or chemiluminescent reactions. Recently chemiluminescence has been proposed as a valid alternative to radioactive or colorimetric methods in a variety of assays using enzymes as labels, due to its sensitivity, reliability and possibility to give quantitative results (Musiani et al., 1991a,b; Dubitsky et al., 1992; Holtze et al., 1992; Martin et al., 1995; Lorimier et al., 1996).

In situ nucleic acid hybridization assays provide an important tool for studying molecular information inside individual cells within a tissue or cell population, thus allowing to precisely identify cell types containing or expressing nucleic acids of interest and to correlate the molecular information with the morphological structure (Wilcox, 1993). In situ hybridization for the detection of specific nucleic acids in cells and tissues are extensively used in research and routine laboratories and they have applications in a variety of bio-medical fields including oncology, microbiology, virology, transplantation and inherited disorders (Diamandis, 1988; Matthews and Kricka, 1988; Leary and Ruth, 1989; Pollard-Knight, 1991; Wenham, 1992; Rapley and Walker, 1993).

In in situ hybridization, labeled nucleic acid probes are used and different labels and label detection methods are available. The first assays used a radioactive phosphorus-32 label, which has the disadvantage of a

relatively short half-life, thus placing limitations on the routine use and commercialization of the probes (Kricka, 1985). Other drawbacks are the possible health hazards, the disposal of radioactive waste and long periods (days) to develop the signal. Recently, many substances for direct or indirect labeling of nucleic acid probes have been proposed as nonisotopic alternatives to radioactive labels. Direct labels, including enzymes, fluorophores and chemiluminescent molecules covalently attached to the probe, are directly detectable, while indirect labels require secondary recognition after hybridization and include biotin and other haptens (Misiura et al., 1990). In in situ hybridization assays, indirect labels are preferred for their increased sensitivity and biotinylated and digoxigenin-labeled probes are the most widely used. These probes are revealed by streptavidin or anti-digoxigenin antibody, respectively, with a covalently attached signaling group that is usually alkaline phosphatase (AP) or horseradish peroxidase (HRP). These enzymes (AP and HRP) can be revealed using different detection techniques mainly including colorimetry, fluorescence and more recently chemiluminescence. With regards to colorimetric detection, for AP many chromogenic substrates have been developed; the most widely used in in situ hybridization is 5-bromo-4-chloro-3-indolylphosphate (BCIP) which, after dephosphorylation and subsequent reaction with the dye nitroblue tetrazolium (NBT), produces a dark blue colored precipitate (McGadley, 1970). For HRP, a wide assortment of colorimetric substrates is available and the most commonly used include 3-amino-9-ethylcarbazole (AEC) and 3,3'-diaminobenzidine (DAB). Anyway, in these last years, it has been demonstrated that chemiluminescent detection systems which combine the use of an enzyme and a substrate that is converted to a light-emitting product, have the highest potential sensitivity; moreover chemiluminescent systems can give precise and accurate quantitative results since the luminescent signal intensity is proportional to enzyme concentration present in the reaction (Lamarcq et al., 1995). The recent development of novel chemiluminescent substrates for alkaline phosphatase which include different derivatives of adamantil 1,2-dioxetane phenyl phosphate (Bronstein and Kricka, 1989; Bronstein et al., 1989, 1990; Schaap et al., 1989; Beck and Köster, 1990) has led to an increased detection sensitivity in the analysis of nucleic acids. These substrates have a glowing kinetics with a steady state emission which permits easier handling and analysis of the samples and are very sensitive being able to reveal as few as 1.6 zeptomoles of the enzyme (Bronstein et al., 1990). Recently, horseradish peroxidase-catalyzed chemiluminescence has been used as a detection system for nucleic acids analysis. This is due to the development of enhanced chemiluminescent reagents containing substances which enhance the light production deriving from the HRP-catalyzed oxidation of luminol by hydrogen peroxide (Matthews et al., 1985; Thorpe and Kricka, 1986, 1987).

Continuing improvements in chemiluminescent substrates have recently been matched by new developments in photon imaging instrumentation such as high performance luminographs based on a CCD videocamera or high dynamic range pick up tube (Saticon) combined with a video amplifier. These instruments not only allow a quantification of emitted light at a single photon level but also permit localization of the chemiluminescent emission on a target surface (Scott and Inaba, 1989; Wick, 1989; Hooper and Ansorge, 1991; Bräuer et al., 1993; Roda et al., 1996). Moreover, connecting the luminograph to an optical microscope, it is possible to localize the light emission inside tissues or cells (Hiraoka et al., 1987; Mueller-Klieser et al., 1988; Hawkins and Cumming, 1990; Lorimier et al., 1993; Mueller-Klieser and Walenta, 1993; Musiani et al., 1996a,b).

Samples

Several specimen types have already been used for chemiluminescence in situ hybridization especially for the detection of viral genomes such as Cytomegalovirus, *Herpes simplex*, Parvovirus B19, Human Papillomavirus DNAs and the procedure is currently performed in pathology laboratories. Different kinds of specimens such as cellular smears and frozen or paraffin embedded tissue sections can be analyzed. Cells and sections have to be placed on pretreated silanated slides. The thickness of the sections must be accurately defined in order to obtain optimized results; the currently used cryostats obtain 5-8 μm thick sections suitable for this purpose. The chemiluminescence in situ hybridization is currently performed using digoxigenin-labeled DNA probes; once the hybridization reaction has occurred following conventional protocols, incubation with an antibody against digoxigenin, labeled with AP or HRP, is performed, then the signal from the target is revealed with the appropriate chemiluminescent substrate and imaged. Alternatively, biotinylated DNA probes are used; after in situ hybridization reaction, a streptavidin-biotinylated HRP complex is used to amplify the signal, which is then detected with the chemiluminescent substrate.

Chemiluminescent detection

The chemiluminescent detection systems up to now mainly rely on HRP- or AP-chemiluminescent substrates such as luminol-based reagents (i.e. ECL system: luminol/H_2O_2/enhancer) and dioxetane phosphate-based reagents (i.e. CSPD, CDP-star, Lumi-Phos Plus), respectively. The luminol based reagents such as ECL substrate for HRP are well standardized reagents for HRP activity chemiluminescence analysis. For this purpose it is sufficient to add a 20 μl drop over the sample, then to put the samples under the microscope connected with the videocamera and acquire the light signal in few minutes. In fact the light emission kinetics is of a glowing type with a plateau stable for at least 10

minutes and whose intensity is proportional to the enzyme activity. Concerning AP activity chemiluminescent detection, commercially available dioxetane phosphate-based substrates are successfully used reaching a very high detectability. The kinetics is of a glowing type also in this case, but it is slower. Anyway it is sufficient to add 20 μl of substrate solution and acquire the light emission after 15 minutes incubation. This time is necessary to reach a steady-state light emission. In both cases the emission is in the visible light (495 nm for HRP and 530 nm for AP).

Instrumentation

The instrument suitable for ultra-sensitive and quantitative optical microscopy-chemiluminescent imaging of chemiluminescence in situ hybridization is composed by the following parts:

a) a conventional light microscope with a simple lens coupling system. The optics must be selected in order to obtain a magnification factor of at least 360 with a 40x objective and a minimum spatial resolution of 1 mm. The lens must have the highest numerical aperture compatible with focal aberration and depth of field, thus reducing the potential loss of light deriving from the chemiluminescent reaction. The microscope should also be provided with a micrometric system to automatically adjust the position of the sample, allowing to find exactly the same field of view after slide removal for washing and handling procedures. The microscope has to be enclosed in a dark box to prevent contact with the external light during the measurements.

b) a videocamera. The best camera format in terms of sensitivity, dynamic range, spatial resolution should be a cooled CCD intensified with one or two stage image intensifier. The thermal noise should be as low as possible and this is achieved using a cooled system. The camera should be calibrated with a light standard such as a liquid scintillation radioactive isotope (^{14}C, ^{3}H) or an electronically controlled light emitting diode (LED).

c) a software for the camera management and the image processing and analysis. Concerning the camera management, the mode of integration of light should be chosen to have a wide dynamic range without saturation of the light sensitive elements. It should be possible to acquire the light signal for different intervals of time with the possibility to have a multiframe time-dependent image. Besides chemiluminescent images, the camera has to be able to acquire and store light transmitted images in order to properly identify the morphological structure of the sample. A series of corrections should be performed after the acquisition of the images such as background, gamma, geometric, flat field, defect corrections.

With regards to image processing and analysis, a series of mathematical functions should be available to improve the quality of the chemiluminescent and live images. Pseudocolor and overlay functions are necessary to superimpose chemiluminescent image to light transmitted image in order to appropriately localize the light emission from the target. A quantitative analysis of the light emitted in a given area of the sample (pixel) should be performed. It should also be possible to standardize the above mentioned parameters to allow a precise, accurate and reliable comparison of specimens analyzed in different sets of experiments. High quality color prints of the images with appropriate comments, according to the guidelines of good laboratory practice and method standardization should be carried out (Fig. 1).

The instrumental system to detect positive signals after in situ hybridization generally operates in three

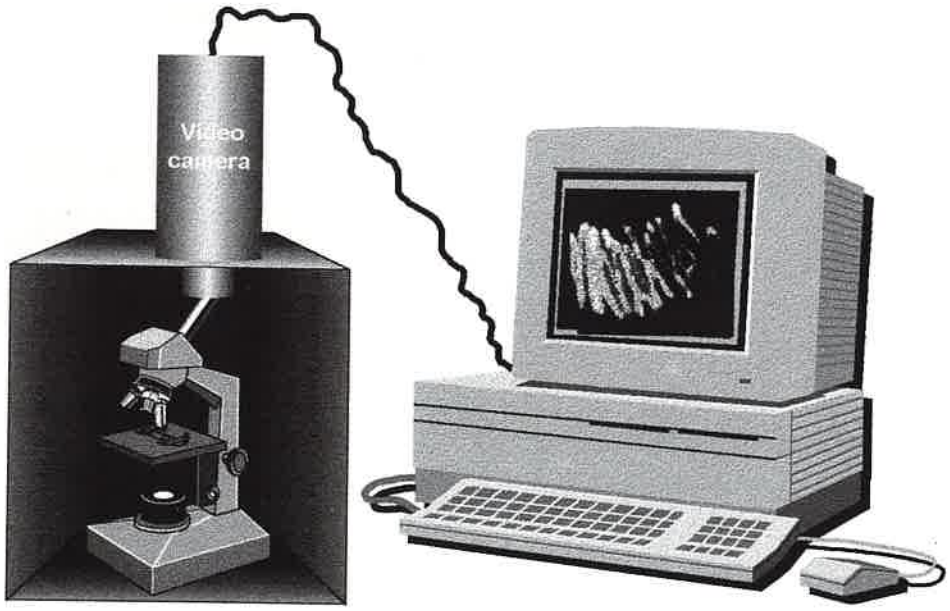

Fig. 1. Imaging system for the analysis of chemiluminescence in situ hybridization. A high performance low light level imaging apparatus is connected to an optical microscope and to a personal computer for quantitative image analysis.

steps: firstly tissue structures and cells are recorded in transmitted light then the luminescent signal is measured with an optimized photon accumulation lasting one minute; and then, after a computer elaboration of the luminescent signal with pseudocolors corresponding to the light intensity, an overlay of the two images on the screen provided by the transmitted light and by the luminescent signal allows the spatial distribution of the labeled probe to be localized and evaluated (Fig. 2). Digital images of the light emission from cells or tissue specimens are optimized at about 2 sec intervals integration time for one minute total accumulation time. The light emission from each cell can be quantified by defining a fixed area and summing the total number of photon fluxes from within this area. Negative control specimens are also analyzed, providing threshold background levels. Corrections for instrumental background and flat field variations can be automatically performed and the net light signal is then calculated by subtracting the samples' values with threshold background values and expressed as integrated photons/sec/area.

Discussion

The main advantages of chemiluminescence in situ hybridization are mainly the sensitivity, the quantification of the data, the objectivity of the evaluation and the digital imaging of the results.

Chemiluminescence in situ hybridization proved to be very sensitive, being able to detect as few as 10 to 50 viral genome copies in infected cells using both biotynilated probes and digoxigenin labeled probes with HRP and AP chemiluminescent detections respectively. Positive chemiluminescent signals were in fact obtained in HeLa cells which are known to contain about 10 to 50 integrated genome copies of human papillomavirus (HPV) 18 (Lorimier et al., 1996; Musiani et al., 1997). Chemiluminescence in situ hybridization has proved more sensitive than in situ hybridization followed by colorimetric detection and almost as sensitive as in situ hybridization followed by ^{35}S autoradiography; in fact, the chemiluminescence positivity of the results obtained from the detection of HPV DNA in HeLa cells was not obtained with colorimetric detection (Lorimier et al., 1993; Musiani et al., 1997) while chemiluminescent signal resolution was comparable to that provided by ^{35}S autoradiography (Lorimier et al., 1996). Moreover with chemiluminescence in situ hybridization to detect B19 parvovirus DNA in several samples of bone marrow cells, all the positive specimens from patients with a diagnosed B19 infection proved positive with a higher number of positive cells/specimen in comparison with colorimetric detection, thus permitting an easier evaluation of the sample (Musiani et al., 1996b).

Chemiluminescence in situ hybridization allows the quantification of the results; in fact results on smears of Caski and HeLa cells (which are known to contain 500-600 copies of HPV 16 DNA and 10-50 copies of HPV 18 DNA respectively) demonstrated that the luminescent signal changed in proportion to the known numbers of viral genome copies per cell (Musiani et al., 1997). In addition, studying the presence of CMV DNA in infected cells, increasing values of emitted photons/cell

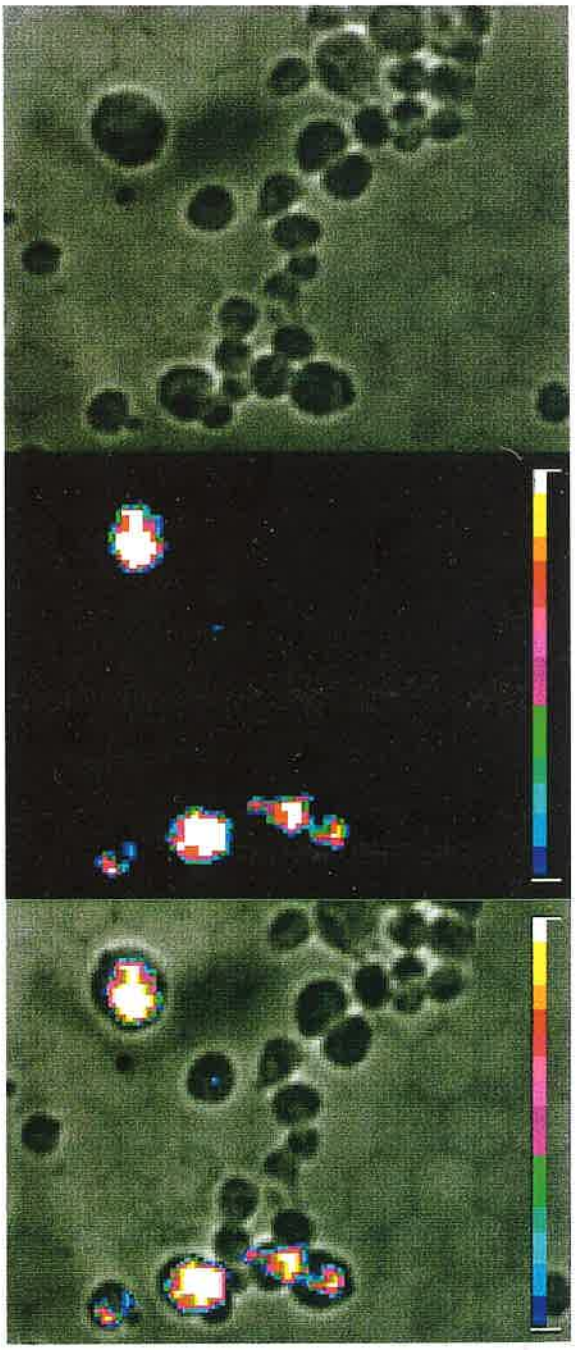

Fig.2. Chemiluminescence in situ hybridization revealing Cytomegalovirus (CMV) DNA in human fibroblasts infected with CMV. Cells were tripsinized, smeared and fixed at 96 hours after infection. From top to bottom: Live image; luminescent signal; overlay of the live image and luminescent signal.

corresponding to the presence of hybridized CMV DNA, could be found in cells fixed at 48, 60, 72 and 96 hours after infection, following the CMV replication cycle (Musiani et al., 1996a).

With chemiluminescence in situ hybridization, since the positive signal is considered the one above threshold values, an objective evaluation of the results could be achieved without any training at the microscope to read the slides and so doubts about positive or negative results are minimized. Chemiluminescence in situ hybridization moreover offers a permanent record of the reactions as all the images of the samples are stored in the computer and these images can be printed or sent for an evaluation in other laboratories using floppy disks or other computer networks.

In conclusion, the chemiluminescent in situ hybridization assay can be a useful tool for a sensitive and specific diagnosis of viral infection and for the detection and study of specific genic sequences inside the cells. The use of the chemiluminescent in situ hybridization assay, which can be applied to cell smears, archival frozen and paraffin embedded tissue samples, may also be promising for an estimation and quantification of nucleic acids present in tissue samples or cellular smears and for imaging gene expression in cells, provided a strong standardization of the methods, reagents and samples.

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