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Qualitative autoradiography with polycarbonate foils enables histological and track analyses on the same section

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Abstract

Neutron autoradiography is an imaging methodology that enables analysis of the spatial distribution of heavy ion emitters in a given material. In particular, it allows localization of ^{10}B in a tissue section put in contact with a nuclear track detector. Boron imaging is essential when considering boron neutron capture therapy as an option for treating cancerous tumors. A description of the autoradiography method is presented together with specific characteristics and technical details developed in our laboratory. We propose a new mounting technique to compare autoradiography images with the same section that gave rise to the latent tracks. The solid state nuclear track detector is polycarbonate, because it can be processed rapidly to obtain the autoradiographic results. It is a transparent material, which allows visualization of the sections mounted on it. Tissue can be removed easily and background is minimal.

Key words: BNCT, boron imaging, histochemical localization, neutron autoradiography, SSNTD

Recently, a binary radiotherapy modality, boron neutron capture therapy (BNCT) has been developed to treat tumors that respond poorly to conventional radiotherapy (Barth et al. 2009). A neutron beam interacts with boron injected into the patient. A capture reaction then is produced between slow neutrons and ^{10}B atoms; as a result, alpha particles and lithium nuclei are emitted. Both particles have high linear energy transfer (LET) and deposit their energy in the immediate vicinity of the reaction position, within a range of approximately 5–9 μm, which is roughly equal to one cell diameter. These interactions produce highly localized damage in tumor cells (Coderre et al. 2003). The compounds employed most commonly are boronophenylalanine (BPA) and sodium mercaptoundecahydrododecaborane (BSH) (Soloway et al. 1998, Barth et al. 2005).

When BNCT treatment is considered, preferential uptake of ^{10}B by the tumor cells is required. In this way, healthy tissue ideally is not affected by irradiation with thermal neutrons. The final effect of the treatment depends heavily on a higher concentration of boron in the tumor compared to surrounding normal tissue. Knowledge of ^{10}B distribution in tumors, in neighboring tissue, and in organs such as kidney or liver, is vitally important when planning this kind of therapy. If the local distribution of boron in tissue can be determined quantitatively, understanding of the mechanisms involved in tumor uptake of boron compounds would be enhanced (Wittig et al. 2008). The boron concentration usually is different for each tissue owing to different metabolic activities. Therefore, its measured concentration in the whole tissue sample

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may differ with the percentage of active tumor cells with respect to necrotic and normal tissue. A rapid method that reveals this difference would be helpful for evaluating the tumor response.

Autoradiographic techniques with solid state nuclear track detectors (SSNTD) (Abe et al. 1986, Durrani and Bull 1987) have demonstrated their usefulness for determining boron distribution in biological materials for BNCT. In general, two different tissue sections are needed for autoradiography analysis: one to be irradiated and processed, and another for histological analysis to correlate the autoradiographic findings with the tissue structure (Yanagie et al. 2004, Altieri et al. 2008, Schütz et al. 2011).

We report here the use of the same tissue section for both broad histological study and generation of an autoradiographic image. A general description of the autoradiographic method is presented. Finally, applications of this technique to qualitative evaluation of boron in normal human liver tissue and tumors from a melanoma model are explained in detail.

Materials and methods

Sample preparation

In a biodistribution study, the subject (experimental animal or patient) is injected with a boron compound. After infusion, a time interval is required before resecting the tissue sample to allow the ^{10}B atoms to reach the target cells. This time depends on the transport mechanism of the boron compound, administration protocol etc.

When the tissue is resected, it is fixed immediately in liquid nitrogen or frozen isopentane. The purpose of quick freezing is to preserve a realistic assessment of their distribution by preventing migration of boron atoms from their original sites.

The samples are sectioned in a cryostat to yield sections of about 10 μm and 30–50 μm thickness consecutively; thick and thin sections are obtained alternately. The thinner sections are placed on slides for morphological analysis using hematoxylin and eosin (H & E) staining. The thicker sections are mounted on nuclear track detector foils such as polyallyl diglycol carbonate (PADC) or polycarbonate (Lexan™). Both materials are transparent, so tissue sections can be observed under an optical microscope. Because of the properties of polycarbonate make it easy to handle, we chose Lexan as the track detector for most of our experiments. Tissue sections mounted for SSNTD are covered by another detector foil and irradiated as explained below (see *Autoradiography*). After irradiation, the tissue is stained with hematoxylin alone or with H & E. The detector is not altered by the staining, even though the section remains permanently colored. A chemical attack, or etching, is required to enlarge the nuclear tracks at the light microscope level (see *Autoradiography*). After etching, the detector surface does not show irregularities associated with staining alterations. The detector does not remain stained and the surface is attacked homogeneously. Moreover, comparing a detector that had a colored tissue section on it with another that had an unstained one, the size, shape and density of the etched tracks were unchanged; this indicates that the latent tracks are not affected by the staining.

Other stains were tested, such as Grenacher's alum carmine or gentian violet, but hematoxylin or H & E provided rapid and permanent staining. For other purposes, such as high resolution quantitative autoradiography (HRQAR) (Solares and Zamenhof 1995), a paler solution such as Grenacher's carmine is preferred to distinguish better cells and nuclear tracks, which are observed simultaneously.

The tissue section on the detector is photographed at low magnification (e.g., objectives $1.25\times/0.035$ or $2.5\times/0.075$), to document the whole section. Photos at higher magnification also can be obtained (e.g., 10 or $40\times$).

Tissue eventually must be removed from the detector. PADC is not affected if submerged in xylene, but Lexan is. After several trials, a trypsin-EDTA solution (0.25–2%) was chosen for this purpose. After tissue removal, the foils are cleaned with 96% alcohol after which they are ready for processing.

Autoradiography

The detector-tissue-detector assemblies are irradiated in a thermal neutron flux to initiate prompt neutron capture reactions with the boron atoms in the tissue. The irradiations are carried out at the RA-3 reactor (Ezeiza Atomic Center, Buenos Aires, Argentina). This is an open-pool reactor with an isotropic and uniform neutron flux of $(9\pm1)\times10^9$ n cm^{-2} s^{-1} (Miller et al. 2009).

The alpha particles and lithium ions that are produced cause damage trails in the detector foils. These latent tracks can be revealed at the light microscope scale by treating the plastic foils with an alkaline etching solution (Fleischer 1975). As a result, the autoradiographic image is formed on the detector foil. The covering detector foil serves as both a tissue protector and a back-up of the autoradiography image.

Depending on the neutron flux, boron concentration and etching conditions, the tracks in the autoradiographies could be separated enough to allow individual counting. Track density can be translated to ^{10}B concentration using a calibration system. The quantitative analysis has been described previously (Portu et al. 2011a,b). By varying the irradiation conditions, it is possible to obtain autoradiographic images with a high density of tracks to evaluate qualitatively differences in shades of gray, thus giving information about the spatial distribution of boron.

Qualitative analysis of tissue samples from BNCT protocols

To provide some examples of the methodology, samples from different BNCT protocols were analyzed: 1) samples of normal human liver from patients injected with boronphenilalaninefructose (BPA) and preserved in liquid nitrogen (Cardoso et al. 2009) and 2) samples of melanoma tumors obtained from NIH nude mice implanted with a cell line of human melanoma MELJ (Carpano et al. 2010). These samples were injected with BPA at a dose of 350 mg/kg body weight (bw) and animals were euthanized 2 h post-administration.

All samples were sectioned at 30 μm in a cryostat and mounted on Lexan foils (15 mm × 15 mm × 250 μm thick). The assemblies were exposed to thermal neutrons at a fluence of 10^{13} n cm^{-2} to obtain a high density of tracks. A reference system is needed to correlate the location of tissue evaluated with the position of the tracks appearing later in the autoradiographic image. Before dismantling the irradiation assemblies, four reference points were drilled with a drill bit (diameter approximately 0.5 mm).

While still on the Lexan, the sections were stained with hematoxylin (BIOPUR, ANMAT, Cert. No. 003112). The detector foil was attached to a slide at a defined position, then photographed using a CCD camera (Axioplan, Carl Zeiss, Germany) at different magnifications. The samples were examined and the zones of interest were delimited by using the reference points drilled earlier.

After removing the tissue with trypsin, the foils were treated chemically with PEW solution (30 g KOH + 80 g ethyl alcohol + 90 g distilled water) at 70° C for 6 min. New pictures of the foils were obtained and compared with the earlier images. The position of the slide on the microscope plate was reproduced to capture the same field, while preserving the tissue orientation in the picture. Using the reference marks, the coordinates of the nuclear tracks could be related to the region of interest in the tissue.

In cases where a more comprehensive histological analysis is necessary, consecutive 10 μm sections could be mounted on slides for observation.

Results

Because Lexan is a transparent polymer, observation of tissue sections under the light microscope is as feasible as if it were mounted on a slide. Moreover, H & E do not color the polymer, although the tissue remains permanently stained.

A high degree of track overlapping occurred under the irradiation and etching conditions described here; thus, the autoradiographic image could be observed clearly at low magnification.

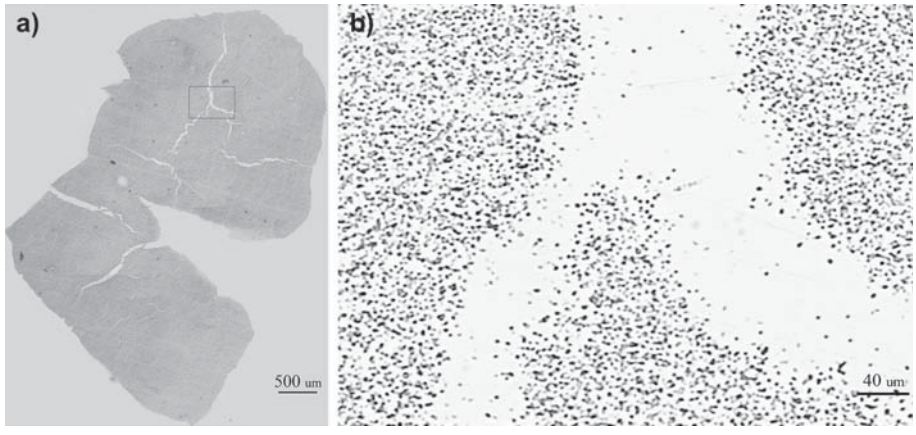

Fig. 1. Autoradiography of a normal human liver slice (~30 μm; BPA at a dose of 300 mg kg^{-1}; 10^{13} n cm^{-2}). a) 2.5×. b) 40×. The square in (a) indicates the position of the enlarged region in (b).

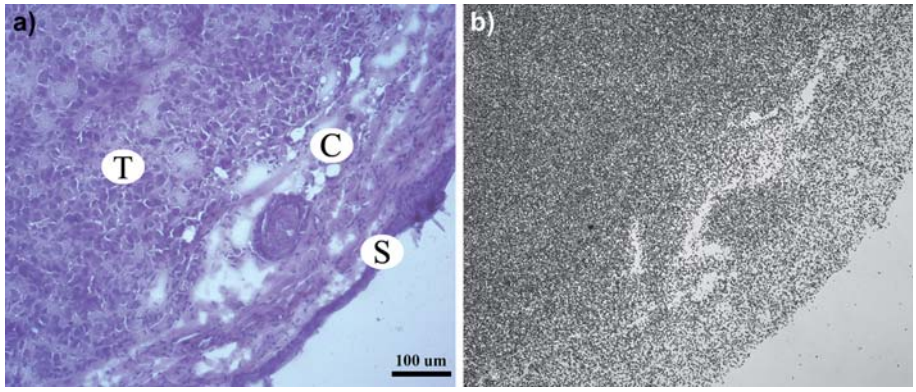

Fig. 2. a) Hematoxylin stained melanoma tumor cryosection. 10 ×. b) Autoradiographic image corresponding to the section showed in (a) at the same magnification. T, tumor; C, connective tissue; S, skin.

An example of the procedure for human liver samples is shown in Fig. 1a. A region of Fig. 1a at higher magnification is shown in Fig. 1b. Scratches on the sections are revealed as paths without tracks. The apparently continuous image actually is formed by individual tracks that can be seen clearly in Fig. 1b.

Figure 2a,b illustrates the procedure for the melanoma experimental model. In Fig. 2a, a hematoxylin stained tumor section is shown. The viable tumor can be differentiated readily from the surrounding skin. Figure 2b was obtained following the steps described above. Substantial differences in shades of gray between the two regions indicate that the tumor has accumulated a greater amount of ^{10}B.

Discussion

We have given examples that demonstrate the ability of boron imaging to provide valuable information about the spatial distribution of ^{10}B in different regions of tissue samples. We have shown that the structural characteristics of the tissues are clearly discernible in the autoradiographic images.

Using our technique, the boron distribution observed by autoradiography reflects the histology of the same section that was used to produce the track image; this constitutes the main advantage of the method presented here. When analyzing boron distribution at the microscopic level, any shift between the histological image and the autoradiograph increases the uncertainty about the spatial localization of specific structures of interest. The alternative use of adjacent slices may present significant differences in the cutting plane depending on the slice thickness and this can lead to diminished resolution.

From our experience, although the tissue is destroyed after the etching process, the comparison between autoradiographic images and the prior photographs is perfectly maintained; however, if further analysis is required, consecutive histological sections could be observed.

We achieved excellent results with the methodology presented here. Besides its simplicity, no extra materials beyond those found in any conventional histopathology laboratory are required. Our method can be used to obtain the following information: the histological detail of the material analyzed; location of the nuclear tracks, which eventually could be converted to boron concentration, in the same slice that was analyzed histologically rather than an adjacent section; a sensitive comparison between tumor and neighboring tissues such as necrotic or healthy tissue, which permits a reasonable estimation of dose, information that is essential for treatment planning to limit exposure in surrounding normal tissue.

This information enables a microdosimetric analysis, which is essential for a highly localized radiation therapy such as BNCT. Understanding the boron distribution at the microscopic level is necessary to understand its radiobiological effects and to evaluate the clinical efficacy of BNCT. In particular, preservation of surrounding skin is of vital importance during the treatment of melanomas. The analysis, however, can be applied to any tissue selected for BNCT treatment.

The technique described here can be extended easily to analysis of biological samples that contain other heavy ionizing particle emitters in addition to ^{10}B such as ^{238}U; only the etching conditions must

be revised owing to potential differences in the ranges of the particles.

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