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John F. Harms  
*Messiah College*, jharms@messiah.edu  
L. R. Budgeon  
N. D. Christensen  
D. R. Welch

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This result allowed us to conclude that arsenite-induced apoptosis of this cell line was through the activation of caspase-3, although it remains unclear what reaction component(s) were modified by boiling. The mechanism of increasing sensitivity by the boiling method needs to be studied further.

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Address correspondence to Dr. Lizhi Zeng, H.L. Snyder Medical Research Institute, 1407 Wheat Road, Winfield, KS 67156, USA. e-mail: lzeng@snyderrf.org

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Lizhi Zeng and Larry D. Smith
H.L. Snyder Medical Research Institute
Winfield, KS, USA

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Maintaining GFP Tissue Fluorescence through Bone Decalcification and Long-Term Storage

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Decalcification of bone is required for frozen or standard histological sectioning; however, acidic decalcification solutions abrogate the fluorescence of tissues expressing enhanced GFP. In addition, long-term storage of fluorescing tissues from in vivo studies necessitates maintaining GFP fluorescence in a solution that does not compromise tissue and cellular integrity.

The spread of metastatic cancer to skeletal sites is a grim complication frequent in breast, prostate, and lung cancers. In particular, the incidence of breast cancer metastasis to bone has been estimated to be as high as 85% (2), causing osteolytic lesions that result in pathological fractures, spinal cord compression, and hypercalcemia. Why

Figure 1. Fluorescence of GFP-tagged breast cancer metastases is maintained through decalcification and frozen sectioning of murine hind limb bones. (A) Fluorescence microscopy of whole femur and proximal tibia following 4% paraformaldehyde fixation. Bar = 1 mm. (B) Fluorescence following 14 h incubation in 0.5 M EDTA in CMF-PBS, immediately preceding frozen sectioning. (C and E) Bright-field photomicrographs of frozen sections. Tumor (T) has filled medullary canal but has not crossed the ephysyeal growth plate (P) into distal normal marrow (M). Since the ephysyeal growth plate is normal murine tissue, it does not fluoresce. Normal murine tissue also exists between tumor cells, and some spaces are the result of tissue sectioning artifact. (C, Bar = 1 mm; E, Bar = 0.1 mm). (D and F) Corresponding fluorescence microscopy reveals fluorescing tumor tissue replacing marrow of the medullary canal.
breast cancer exhibits significant predilection for bone is unknown. To model skeletal metastasis in vivo, we engineered metastatic human breast carcinoma cell lines (MDA-MB-435 and MDA-MB-231) to constitutively express GFP (4). Intracardiac injection of cells into the left ventricle of female athymic mice produces widespread skeletal metastases, localized predominantly to the trabecular regions of bones including femur, proximal tibia, proximal humerus, and lumbar vertebrae. The utility of GFP-tagging for the detection of metastases and tracking of single cells in vivo has been clearly demonstrated in several models (3,5,7) including bone metastasis (6,8–11). Bone metastases may be easily identified in whole bone without laborious sectioning or radiographic detection that customarily requires decalcification of at least 50% of the mineralized bone (1). However, histological sectioning is required to determine the position of metastases at the microscopic level. Paraffin or frozen sectioning of bone without fixation and decalcification often causes shattering of the calcified tissue and contributes to significant wear or chipping of blades. While 4% paraformaldehyde fixation of GFP tissues maintains fluorescence, decalcification methods employing acidic solutions quickly abrogate fluorescence.

Metastatic MDA-MB-435 cells were transfected with pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA, USA) by electroporation (GenePulser, Bio-Rad, Laboratories, Hercules, CA, USA; 220 V, 960 μFd, ∞Ω). The brightest 25% of the neomycin-resistant fluorescently tagged cells were sorted using a Coulter EPICS V cell sorter (Beckman Coulter, Fullerton, CA, USA). Cells were introduced into athymic mice by either mammary fat pad or intracardiac injection. Fresh primary tumors fluoresced and continued to fluoresce following fixation in freshly prepared 4% paraformaldehyde (4°C) for 24–48 h. Tissues were examined using a Leica MZFLIII dissection microscope, equipped with GFP2 filter set (Leica, Deerfield, IL, USA). Samples were subsequently exposed to common decalcification solutions including CalEX® (Fisher Scientific, Pittsburgh, PA, USA), 10% sodium citrate/22.5% formic acid, and 0.5 M EDTA in calcium and magnesium-free Dulbecco’s PBS (CMF-PBS) (pH 7.8, 4°C). While fluorescence was eliminated following incubation in the acidic solutions (CalEX and sodium citrate/formic acid), 0.5 M EDTA maintained tissue fluorescence (Figure 1, A and B). Next, to determine the minimum incubation time sufficient for decalcification, two hind limbs, dissected free of soft tissue, were incubated in 10 mL 0.5 M EDTA (4°C) and removed at various time points including 6, 12, 18, 24, 36 and 48 h. Bones were then mounted in O.C.T. compound (Tissue-Tek, Elkhart, IN, USA) and frozen-sectioned. Bones decalcified for 18 h contained limited calcified deposits, as determined by blade sound and feel during frozen sectioning, while 24-h treatment achieved complete decalcification and eliminated blade wear. Fluorescing skeletal metastases were readily visible in frozen sections by fluorescence microscopy (Figure 1, C–F).

Historically, extended archiving of fluorescently tagged tissues, including GFP-labeled cells, has also been problematic. Freezing of tissues may protect fluorescence but can introduce freezing artifacts. While 4% paraformaldehyde maintains fluorescence during fixation, long incubations can deteriorate fluorescence, as can extended storage in 70% ethanol following fixation. Nevertheless, retention of paraformaldehyde-fixed tissues (24-h fixation) in only CMF-PBS (4°C) is incapable of preventing tissue autolysis and cellular degradation. Having observed that tissue still fluoresced after several weeks in 0.5 M EDTA at 4°C and that no tissue autolysis was apparent, we tested whether this was a possible long-term storage medium. We have also tested two dilute solutions of paraformaldehyde (0.5% and 1% paraformaldehyde in CMF-PBS) in parallel with 4% paraformaldehyde. Whole murine bones (including skull, mandible, ribcage, vertebral column, pelvis, and limbs) were dissected free of soft tissues, and all bones derived from an individual mouse were combined in a single 25-mL vial. Vials were filled with 4% paraformaldehyde (approximately 18 mL) for

![Figure 2. Storage of fluorescent tissues in 0.5 M EDTA or 1% paraformaldehyde solutions maintains both tissue fluorescence and morphology.](image-url)
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Vital Stain to Study Cell Invasion in Modified Boyden Chamber Assay

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The capacity of cancer cells to invade basement membrane is a hallmark of metastasis. The modified Boyden chamber assay is often used to analyze and quantify the migratory and invasive potential of cells (5,8). It has a microporous membrane, which separates the two chambers, and is coated with Matrigel™ (BD Biosciences, San Jose, CA, USA) or any other extracellular matrix proteins like fibronectin, laminin, or collagen. Invasive cells seeded in the upper chamber respond to the chemotactic agent in the lower chamber, invade the gel, and migrate to the lower surface of the membrane, whereas noninvasive cells remain in the upper chamber. The chambers are fixed, and cells on the upper side of the filter are removed with a cotton swab. Cells that have migrated to the lower side of the filter are stained [e.g., Diff-Quick (9)] and counted. Quantitation of the results is usually tedious, as cells may not be clearly visible. Also, it is not possible to assess invasion during the incubation period and count cells that have started invading the gel but have not reached the lower surface of the membrane. Numerous technical papers have addressed these difficulties (2,7,9).

We have used a supra vital dye, Hoechst 33342 (Sigma, St. Louis, MO, USA) to stain cells during the invasion assay. This is a vital fluorescent stain that binds specifically to AT-rich nucleolar DNA (1,3,10). It is excited by UV rays and emits blue fluorescence.

In the preliminary experiments, cervical carcinoma (SiHa) cells pulse-labeled for 20 min with Hoechst 33342 at 2 µg/mL showed bright nuclear fluorescence and did not exhibit any toxicity on viability, proliferation, and motility. These observations were comparable to earlier reports (6). The same concentration was used in the subsequent experiments, performed in triplicate. The methodology used was as described previously (4), using 6.5-mm transwell chambers with 8 µm pore size (Corning Costar, Acton, MA, USA).