manufacturer's specifications.

Onto the front of the flashlight we have attached a Kodak® Polymax Filter PC3 (Eastman Kodak, Rochester, NY, USA), and we observe mice through a Kodak Wratten Gelatin Filter 12 (Sigma, Poole, UK). Details of the exact specifications for the filters are available from Eastman Kodak (2). Filter PC3 is no longer available on its own, but an equivalent is available as part of either the Kodak Polymax filter set or the ILFORD Multigrade filter set IV (ILFORD, Cheshire, UK). Any of the higher-contrast filters in the sets will work. Figure 1A illustrates the GFP visualization setup. Filter PC3 restricts the emitted light to around 475 nm, and filter 12 cuts out light below about 500 nm. These filters are not of a high enough quality for high-magnification photomicrographic work; however, for the purpose of identifying GFP expressing animals, they are acceptable. Figure 1B demonstrates the readily visible difference between transgenic and non-transgenic littermates. Using this system, we can identify transgenic animals immediately after birth. Indeed, the optimum time for identification of many transgenic mice is in the first week before hair grows. After the hair has grown, GFP-expressing tissue is still visible (e.g., parts of the nose and feet), but the effect in these areas is not as pronounced.

The system that we have described here fulfills all of our criteria for a GFP visualization system. It is noninvasive. The system is portable and so can be transported and used in an animal care facility with little logistical difficulties. The flashlight identified here is also waterproof and so is easily disinfected. The system requires no warm-up or cool-down period. The components are readily available, and it is relatively inexpensive. The setup described here costs less than $160.

From ethical, time-saving, and financial points of view, it is important to optimize the efficiency of breeding transgenic animals. As more and more laboratories make use of existing GFP transgenic animals, and more GFP transgenics are made, the importance of more effective screening will increase. We feel that the system described here will not be limited to genotyping widely or ubiquitously expressing GFP-transgenic mice but could probably be used for any GFP transgenic organism where the GFP protein is visible. The protein could be expressed either somewhere on the organism's surface or in superficial internal organs visible through the skin.

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Preservation of Fluorescent Protein Activity in Tumor Tissue

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It is now routine to use fluorescent protein technology to monitor developmental brain expression and following tumor growth and metastasis in vivo (1–4). We have explored the use of this technology to assess the impact of radiation and gene therapy using adenovirus on human gliomas grown in nude mice. Tumor cells tagged with GFP in vitro can be implanted intra-cerebrally and subsequently infused with an adenovirus that expresses a complementary fluorescent protein such as DsRed to follow tumor growth and virus transduction. This strategy would not only allow for noninvasive imaging but also one should be able to sort cells isolated from tumor tissue by flow cytometry to assess the effects of therapy on gene expression using microarray analysis. Recently, it was demonstrated that RNA*later*™ (designated the reagent) (Ambion, Austin, TX, USA) not only preserves tissues for subsequent RNA isolation and microarray analysis but also does an excellent job of preserving intracellular structures, making this reagent suitable for proteomics analyses as well (5,6). To preserve the RNA of treated GFP tumors for future microarray analysis, we collected tumors in RNA*later*. Subsequently, upon reexamination of the tissues, we discovered that the activities of EGFP and DsRed (both from BD Biosciences Clontech, Palo Alto, CA, USA) fluorescent proteins were preserved for weeks when stored in the cold, making it feasible to analyze tissues expressing fluorescent proteins days or even weeks after collecting the specimen.

Human glioma U87 cells were expression tagged with EGFP to monitor tumor growth and to assess the effect of radiation on tumor growth. One clone (U87/EGFP-B1) showed little to no difference in growth and radiosurvival compared to the parental U87 cells (Rosenberg et al., in preparation). These cells were then used in subsequent experiments. Female nu/nu mice (Harlan) were injected intra-cerebrally with

U87/EGFP-B1 cells using a stereotactic frame as described previously (7). Forty days after implantation, an adenovirus (AdCMV-DsRed) expressing DsRed was infused intra-tumorally with a dose of 10^9 pfu. Five days after virus infusion, the animals were sacrificed, and the tumors were excised and photographed using a stereomicroscope connected to a CCD camera. Both EGFP fluorescent tumor and DsRed-expressing cells within the tumor were easily detected in fresh specimen using GFP and DsRed filters (not shown). Tumors were cut into smaller pieces and immersed in the reagent and stored at +4°C for subsequent RNA isolation according to the manufacturer's recommendations. When remaining pieces of tumor maintained in reagent were reexamined several weeks later, we found to our surprise that the tumors were still fluorescent. Because the manufacturer recommends storage of tissues at -20°C to preserve RNA for longer periods of time, we also tested whether U87/EGFP-B1 tumors were still fluorescent after several weeks under these conditions. Again, both the GFP and DsRed signals were strong after storage in the reagent for over a week at -20°C (Figure 1A). We reexamined these tumor pieces after 4.5 months, and the fluorescent signals were still strong (Figure 1B).

To better quantify the stability of the GFP signal after storage of cells in RNA*later*, we carried out in vitro experiments with the U87/EGFP-B1 cells. Flow cytometry was done on fresh U87 and U87/EGFP-B1 cells and on cells kept in reagent for 1, 5, and 8 days. We found that the GFP signal was reduced about 80% after storage in reagent at +4°C, but the signal was very stable over the course of the experiment compared to the signal from fresh cells and fluorescent beads (Figure 2). While the reagent preserved about 20% of the signal of fresh cells, this was still about 10 times over the signal obtained with U87 cells treated with reagent. We also noticed a doubling in the autofluorescence of U87 cells kept in reagent compared to fresh cells, which was also stable over this time window.

We reanalyzed the cells kept in reagent at +4°C after 21 days in the microscope and found them to be still fluorescent (Figure 3A). Similarly, U87/EGFP-B1 cells infected with AdCMV-DsRed showed DsRed fluorescence after 11 days in reagent (Figure 3B). These in vitro results support the finding with the tumor specimen that RNA*later* is an excellent preservative for fluorescent proteins.

We conclude that RNA*later* preserves the activity of both GFP and DsRed during longer periods of time at +4°C and -20°C, allowing for repeated

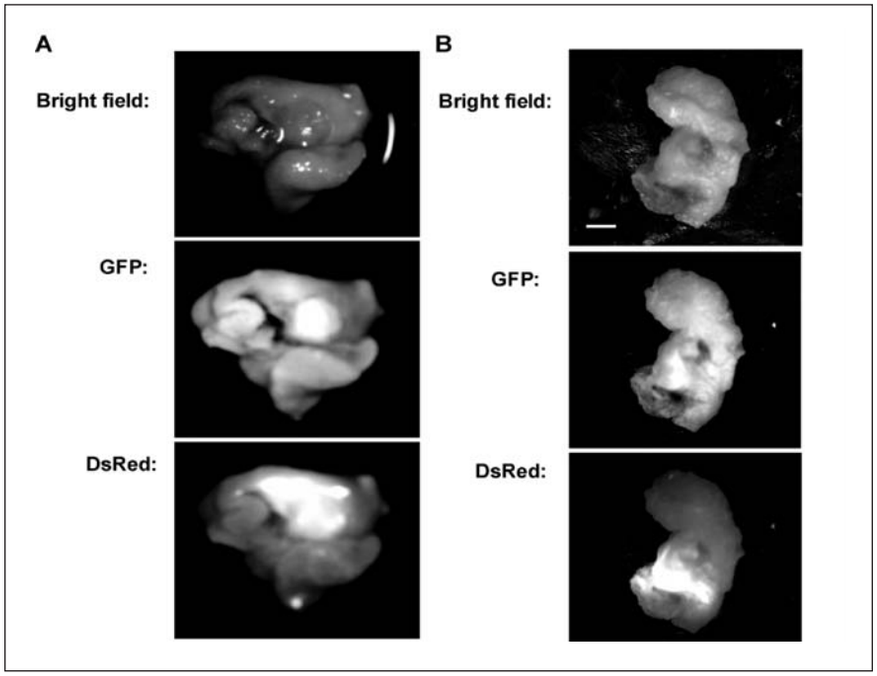

Figure 1. EGFP and DsRed activity in tumors is preserved in RNA*later* after extended periods of time. The panels show pieces of reagent-treated brain tissues surrounding the caudate nucleus with implanted U87/EGFP-B1 cells transduced with AdCMV-DsRed adenovirus. U87/EGFP-B1 (10^5) cells expressing EGFP were intra-cerebrally injected into nude mice using a stereotactic frame (Stoelting, Wood Dale, IL, USA) as described (7,10). Briefly, stereotactic injection was carried out after making a burr hole 2.5 mm lateral and 1 mm anterior to the bregma, inserting a guide-screw directly above the caudate nucleus (11) and injecting the cells in a volume of 3 µL at a depth of 2 mm. Forty days later, AdCMV-DsRed (10^9 pfu) expressing DsRed were infused intra-tumorally in 2 µL at the same site. Five days after virus infusion, the animal was euthanized, and the tumor was removed and immersed in reagent. GFP and DsRed fluorescence was detected in pieces of tumor using a Zeiss SV11 stereomicroscope (Zeiss, Jena, Germany) with a Hamamatsu digital CCD camera C4742-95-12NRB (Hamamatsu, Bridgewater, NJ, USA) and AttoArc2/HB 100 Arc variable intensity microscopy illuminator system (Zeiss) using excitation/emission filters (GFP; 470/525, DsRed; 546/575). Images were captured using AxioVision 3.1 software and processed with Adobe® PhotoShop® 5.0. Pictures were taken of fresh tumor (not shown), 9 (A) and 136 (4.5 months) (B) days at -20°C. The bar in panel B represents 1 mm.

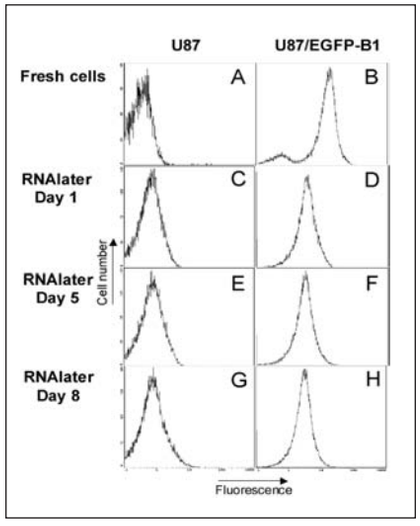

Figure 2. Flow cytometry of fresh and RNA*later*-preserved U87 and U87/EGFP-B1 cells. U87 (A, C, E, and G) and U87/EGFP-B1 (B, D, F, and H) cells were isolated from tissue culture dishes by trypsinization. Flow cytometry was carried out on fresh cells (A and B) and on cells kept in reagent for 1 (C and D), 5 (E and F), or 8 (G and H) days using a Coulter Epics XL-MCL flow cytometer [Ex: 488 nm, with 525 nm (±15 nm) bandpass filter]. The mean fluorescence was obtained, and differences in GFP signal were calculated. Since the GFP fluorescence of fresh U87/EGFP-B1 cells varied slightly from day to day, Immuno-Brite™ Standards Level III fluorescent beads (Beckman Coulter, Miami, FL, USA) were used for normalization.

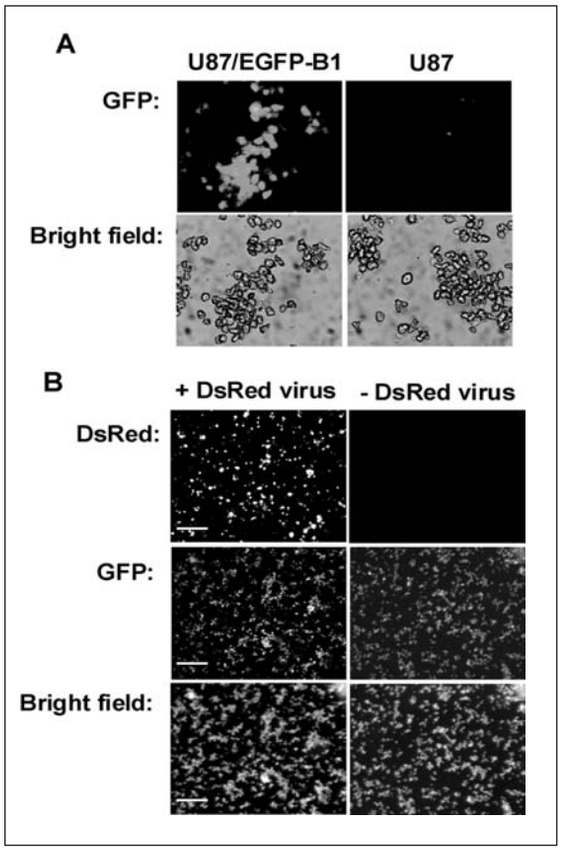

Figure 3. Microscopic images of U87/EGFP-B1 and U87/EGFP-B1 cells infected with AdCMV-DsRed virus preserved in RNA*later*. U87/EGFP-B1 (A) and U87/EGFP-B1 cells infected with AdCMV-DsRed (B) were kept in reagent for 21 (A) and 11 (B) days, respectively, and then examined using (A) a Zeiss fluorescent microscope connected to an Optronics LE-D Hybrid Color digital camera (Optronics, Goleta, CA, USA) or (B) the stereomicroscope described in the legend to Figure 1. The bar represents 250 µm.

microscopic examination of tissues and cells. Although, the GFP and DsRed signal is preserved in reagent, it is reduced about 80% immediately or at least within 24 h. We also found that RNA*later* increases the autofluorescence of U87 cells, probably because of fluorescent additives in the preservative. Thus, tissues and cells expressing GFP and DsRed, and most likely derivatives of these proteins, can be stored in reagent, and cells can be sorted at later times for the isolation of RNA for microarray analysis.

Recently, it was reported that RNA*later* does a remarkable job in preserving proteins and reliably preserve the pathology of tissues (6), and our study on GFP and DsRed now extend these findings to also include the preservation of fluorescent proteins in tissues and cells for long periods of time. Previous studies have demonstrated that animal and *Xenopus laevis* tissues expressing GFP can be frozen or fixed in paraformaldehyde while maintaining the GFP signal (8,9). However, neither freezing nor treatment with paraformaldehyde is expected to preserve RNA as well as RNA*later*. Thus, the use of this reagent would be advantageous when many tumors or tissues are simultaneously processed, and it would logistically be difficult to process multiple fresh samples immediately for flow cytometry or other assays that rely on active fluorescent proteins. Of course, the preservation of tissue culture cells expressing GFP or similar fluorescent protein in reagent for RNA analysis at a later point would also be possible. Because a significant reduction in GFP signal is seen after treatment with reagent, the described method may be limited to cells that express higher levels of GFP.

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