Anti-Cancer Drugs 2000, **11,** pp. 243–248

Clinical report

Scintillation autofluorographic assessment of isotope uptake in human glioma cells grown on microtitration plates using sodium salicylate

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We describe a simple method for detecting [^{35}S]methionine-labeled protein in fixed human astrocytoma cells grown in 96-well microtitration plates using a modified scintillation autofluorographic method. Following isotopic labeling, cells are fixed *in situ* and a solution of salicylic acid in methanol is dried onto the cell layer. The fluorographic image is detected using blue-sensitive X-ray film attached to the base of the plate which, following development, can be quantitated using a scanning densitometer. The relationship between cell number and optical density is linear, and there is a close correlation between the dose–response curves generated by this method and alternative isotopic detection methods and cell counting. This assay provides a suitable alternative to the use of potentially toxic scintillation fluids based on organic solvents like toluene or xylene in chemosensitivity testing of human brain tumors. [© 2000 Lippincott Williams & Wilkins.]

Key words: Chemosensitivity, glioma, isotope uptake, scintillation autofluorography, sodium salicylate.

Introduction

The detection of incorporated radiolabeled precursors in nucleic acid or protein remains an important method for the measurement of cell viability, proliferation and cytotoxicity in cultured cells. The widespread use of 96-well microtitration plates has made it possible to screen large numbers of cell lines against panels of cytotoxic drugs over a wide variety of concentrations with sufficient replication for statistical evaluation. However, these assays are hampered by the technical difficulties in their automation. Cell harvesters can be used, but they are cumbersome and ^{3}H-labeled compounds suffer unpredictable losses at the filter stages.[1] Consequently, manual removal of labeled cells or solubilized protein or nucleic acid is more commonly employed, although this is laborious, time consuming and subject to errors resulting from losses during pipetting.

Freshney and Morgan[1] described a method which can be employed to measure the level of radioisotope incorporated into total cell protein in 96-well microtitration plates *in situ*. Cells are labeled with [^{35}S]methionine and fixed on the plates, and then scintillation fluid is added to each well and dried by centrifugation. A piece of X-ray film is positioned on the base of each plate and, following exposure, developed using standard techniques. The visible light which results from the interaction between the β-particles and the fluors in the scintillation fluid fogs the film, and the density of each spot produced under each well can be determined by a scanning densitometer. However, scintillation fluids based on toluene or xylene dissolve most plastics used in the manufacture of cell culture vessels causing buckling of microtitration plate wells. This interferes with the close apposition of X-ray film and the base of the wells, and causes uneven exposure of the film. Such solvents also leach isotope into the plastic of the plate blurring the image. Sodium salicylate has been used for the fluorographic detection of radiolabeled proteins in polyacrylamide gels,[2] immunoelectropherograms in agarose gels,[3] and thin-layer chromatograms and nitrocellulose blots.[4] The purpose of this paper is to examine the use of sodium salicylate for the fluorographic detection of isotopically labeled proteins in 96-

This work was supported by grants from the Brain Research Trust and the Cancer Research Campaign.

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well microtitration plates and assess its potential in chemosensitivity assays using cells derived from malignant astrocytoma *in vitro*.

Material and methods

Cell culture

U251 MG cells, originally derived from a surgical specimen of human glioblastoma multiforme,[5,6] were the kind gift of Dr Darell D Bigner (Duke University Medical Centre, Durham, NC), and used between passage levels 605 and 620. They were grown in complete growth medium comprising Ham's F-10 medium buffered with 20 mM HEPES and supplemented with 10% selected fetal calf serum (FCS; Gibco/BRL, Paisley, UK). No antibiotics were used during routine culture or in experiments. The cell line was screened on a routine basis (and found negative) for mycoplasma infection using Hoechst 33258 staining using the method described by Chen.[7] Cell counts were carried out using a ZM Coulter Counter calibrated for use with human glioma cells.

Labeling and chemosensitivity experiments were performed in flat-bottomed 96-well microtitration plates (ICN Biomedical, Basingstoke, UK or Bibby Sterilin, Stone, UK). Cells were plated in 100 μl of complete growth medium, the plates sealed with Mylar plate sealers (Titertek, ICN Biomedical) and incubated in a humidified plastic box at 37°C.

Labeling and scintillation autofluorography

L-[^{35}S]methionine (SJ 123; Amersham Pharmacia Biotech, Little Chalfont, UK) in sterile aqueous solution, specific activity 4.37–11.81 mCi/ml (64–448 mCi/mM), was diluted in sterile FCS to a concentration of 20 μCi/ml, aliquoted and stored at −20°C. For labeling the solution was subsequently diluted in Ham's F-10 to the required concentration.

For labeling, growth medium was removed and 100 μl of [^{35}S]methionine in complete growth medium (at a concentration of 2 or 0.2 μCi/ml) added to each well. After an appropriate period of incubation, the Mylar film was removed and the label removed. The plate was then washed twice with HBSS and the cells fixed *in situ* by immersing the entire plate in two changes of methanol. After 10 min fixation, the excess methanol was decanted and the plates air dried. Unincorporated precursors were extracted by gentle washing with ice-cold trichloroacetic acid (TCA; Sigma, Poole, UK) which was prepared as a 10% w/v solution in deionized, distilled water and stored in dark glass bottles at 4°C. The plates were then placed on crushed ice for 5 min after which the TCA was tipped out and extraction repeated. The plates were then washed in running tap water, and dried by immersion in methanol and air drying. The plates were then prepared for scintillation autofluorography using either of the following methods.

(a) Toluene-based scintillation fluid: 50 μl of toluene-base scintillation fluid, NE233 (Nuclear Enterprises) was added to each well on the microtitration plate using a glass semi-automatic dispenser and the plates centrifuged for 1 h at room temperature in a MSE Coolspin centrifuge using microtitration plate carriers (Dynatech, Billingshurst, UK) to produce a flat, uniform film of scintillant in each well.

(b) Sodium salicylate: 50–100 μl of sodium salicylate (Aldrich Chemicals, Gillingham, UK) solution in methanol was added to each well using an automatic pipette (Octapette; Northumbria Biologicals, Cramlington, UK) and the plates centrifuged as described above. Solutions of sodium salicylate were made up freshly as required and handled in subdued lighting conditions.

After either treatment, under a red safety light, X-ray film (X-Omat L; Kodak, Hemel Hempstead, UK) cut from 18 × 24 cm sheets into quarters producing pieces of film approximately 9 × 12 cm, the same size as the underside of a 96-well microtitration plate, was positioned on the underside of each microtitration plate. The film was held in place by a foam rubber sponge and an aluminum pressure plate, and bound with adhesive tape. Each plate was then sealed in a light-tight box together with regenerated silica gel packets to stop condensation and placed at −70°C for 3–5 days.

After exposure, the film was removed and developed for 5 min in D-19 developer (Kodak) which was made up from powder according to the manufacturer's instructions and kept in dark glass bottles at room temperature. The film was then fixed for 5 min in Kodafix solution, diluted according to the manufacturer's instructions, washed in running tap water for 10 min and air dried. For scanning, films were attached to the transparent, moving stage of an EC910 transmission scanning densitometer (Camlab, Cambridge, UK) which had a premarked grid to ensure that the scanning head passed over the centre of the image of each well on the fluorogram. The slit size (0.3 × 3 mm) allowed the scanning of the geometric center of each well (approximate diameter 5–6 mm) The densitometer was fitted with interchangeable filters for Coomassie Blue (550–575 nm) or Amido Black (OB) (600–625 nm). No difference was observed in the quantitative or qualitative pattern obtained with either filter. The baseline reading was set on an

unexposed piece of each film scanned and the span adjusted for full-scale deflection on the darkest well image on each film scanned. Usually, once these had been set, only small adjustments were necessary between different negatives. Scan traces were recorded on a CR500 chart recorder (JJ Instruments, Southampton, UK).

Chemosensitivity assay

Cells were plated out a cell density of 1000–1500 cells/well. Following 48 h incubation at 37°C, to allow the cells to reach exponential growth, the medium was replaced with 100 μl of drug solution. 5-Fluorouracil (Sigma) was dissolved in Ham's F-10 to produce a concentration of 1 mg/ml which was filter sterilized, aliquoted into plastic universals and stored at −70°C. For use in individual experiments, aliquots of the drug were thawed and diluted in complete growth medium and the cells tested over a concentration range of 0.0001–10 μg/ml. Drug dilutions were prepared freshly for each experiment. Drug solutions were renewed 24 and 48 h later to give a total exposure time of 72 h. Wells were rinsed with Hank's balanced salts solution (HBSS; Gibco/BRL) and the cells allowed to recover for 72 h in 100 μl of fresh complete growth medium per well and the plates processed for scintillation autofluorography as described above or for scintillation spectrometry. Here the cells were solubilized by overnight incubation with 100 μl of 1 M sodium hydroxide solution as previously described.[8] The cell extracts were acidified and mixed with 5 mls of PCS (Amersham Pharmacia Biotech) and counted in a polythene vial insert (Bibby Sterilin) in a Nuclear Enterprises NE 1600 scintillation spectrometer. To ensure that the cells remained in exponential growth phase throughout the assay, a separate plate was prepared for each cell line. This was refed each day with fresh medium and six wells were trypsinized each day; the contents pooled and counted.

Results

The use of sodium salicylate as a fluor

Sodium salicylate is highly soluble in water (1 g is soluble in 0.9 ml of water)[9] and initial experiments were carried out to see if aqueous solutions of salicylic acid could be used to detect isotope uptake in 96-well microtitration plates. A major problem was encountered when drying the plates at room temperature overnight. There was a marked tendency for uneven drying in the wells with crystal formation occurring on the sides of the wells rather than evenly over the base (Figure 1a). This was particularly acute when higher concentrations of sodium salicylate (8–64 mg/well)

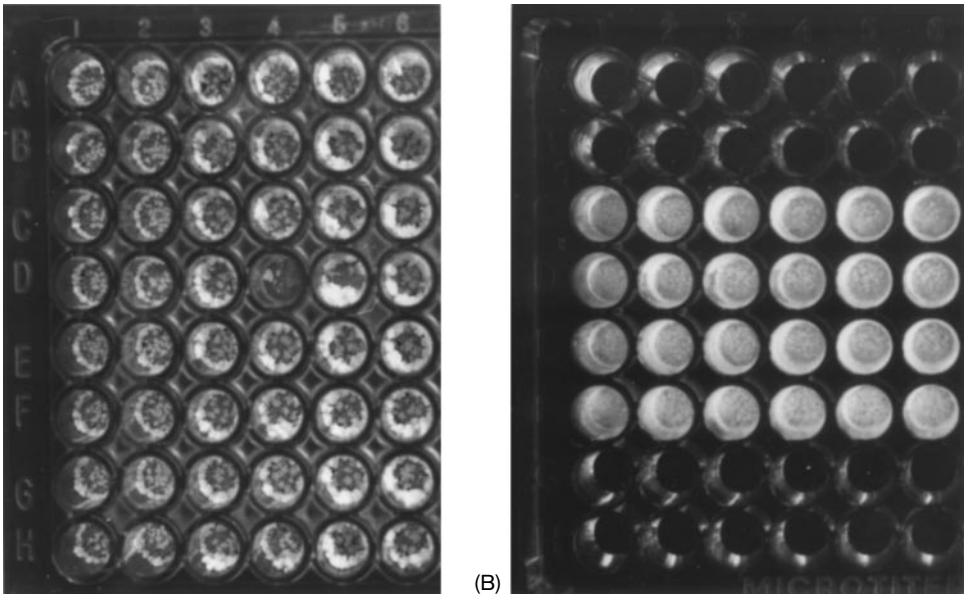

Figure 1. Illustration of two 96-well microtitration plates. Each well of plate A was filled with an 50 μl of aqueous solution of sodium salicylate and the plate dried overnight at room temperature. Note the crystals of sodium salicylate around the sides of the well and that during handling, some of the crystals have become detached leaving the wells empty. The middle four rows of wells in plate B received 100 μl of a saturated solution of sodium salicylate in methanol (approximately 1.1 g/ml w/v sodium salicylate) and dried by centrifugation at 21°C at 1000 r.p.m. as described in the text.

were used. Centrifugation at 1000 r.p.m. for 1–4 h at room temperature did not produce any appreciable drying of the plate. Sodium salicylate is about 10 times less soluble in methanol than water, although a saturated solution of sodium salicylate in methanol still contains 1.1 g/ml of sodium salicylate. When centrifuged for 1 h at 1000 r.p.m., 100–200 μl of such a solution dries to form an even coating in the wells of microtitration plates (Figure 1b). This thin film is rigid enough to withstand the handling used to prepare scintillation autofluorograms. Figure 2 shows a typical developed X-ray film from a plate treated with sodium salicylate in methanol and, for comparison, an equivalent plate treated with toluene-based scintillation fluid. It is clear that there is a better discrimination between wells using sodium salicylate and no evidence of spread of label into the surrounding plastic. The optimal concentration of sodium salicylate was found to be 30 mg/well administered in a volume no greater than 50 μl (Figure 3). Larger volumes of sodium salicylate produced smaller peak heights even at higher concentrations of sodium salicylate because of the unevenness of drying of larger volumes of the solvent.

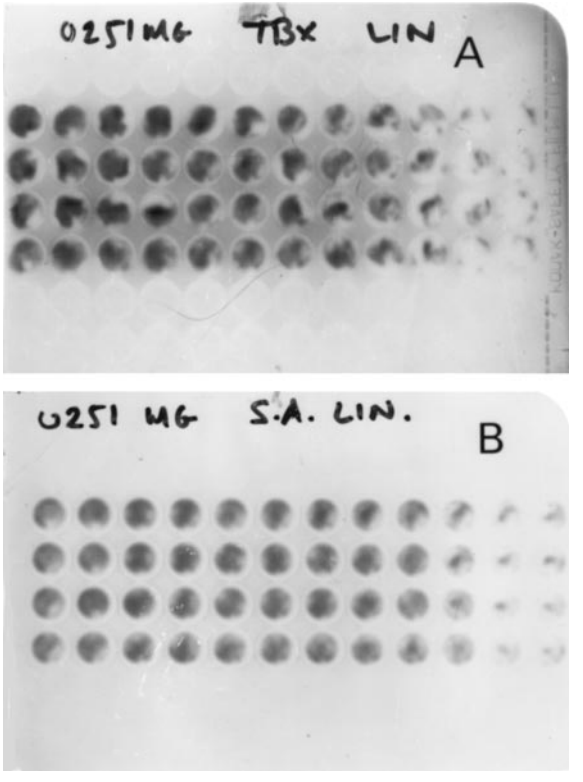

Figure 2. Typical negatives produced as described in the text of two plates with equivalent numbers of U251 MG cells in each plate and identical exposure times: (A) 50 μl of NE233, a toluene-based scintillation fluid, in each well and (B) 50 μl of a saturated solution of sodium salicylate in methanol.

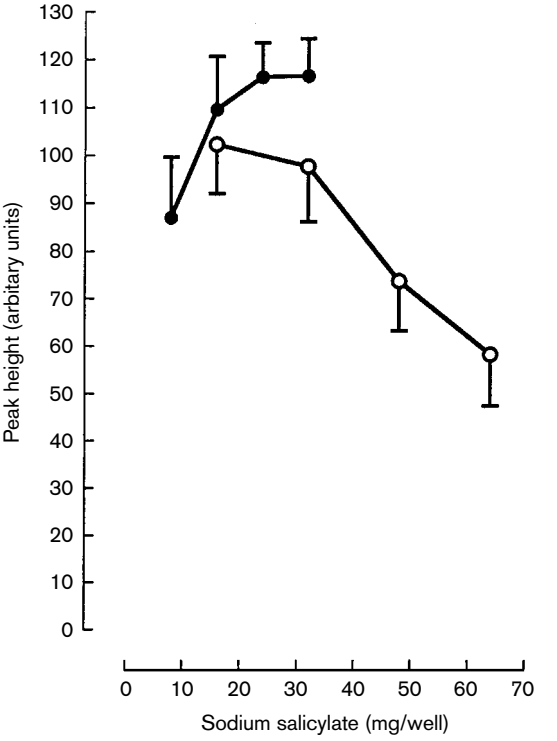

Figure 3. Determination of the optimal concentration and volume of sodium salicylate solution. ●, Experiment 1, total volume 50 μl. ○, Experiment 2, total volume 100 μl.

Linearity of assay

For accurate assessment of cell numbers, it is essential that there is a linear relationship between optical density in the autofluorograms and cell number. In order to determine if this was the case using sodium salicylate, plates were seeded with U251 MG cells at a range of cell densities from 500 to 5000 cells/well. These plates were then incubated for 3 days, labeled with 0.2 μCi/well [^{35}S]methionine for 2, 3 or 4 h and prepared for scintillation autofluorography with sodium salicylate. A further plate was not labeled, but washed with HBSS, and the cells detached with trypsin solution and counted. Following densitometry, the peak heights of wells of each well was determined and calculated as a percentage of the peak height of the initial cell density. Cell counts were calculated as a percentage, taking the initial cell density as 100%. Figure 4 shows the relationship between cell number and peak height. It is apparent that irrespective of time of labeling that the relationship between cell number and [^{35}S]methionine uptake is linear. However, when regression lines are calculated, all the intercepts on the *y*-axis (isotope

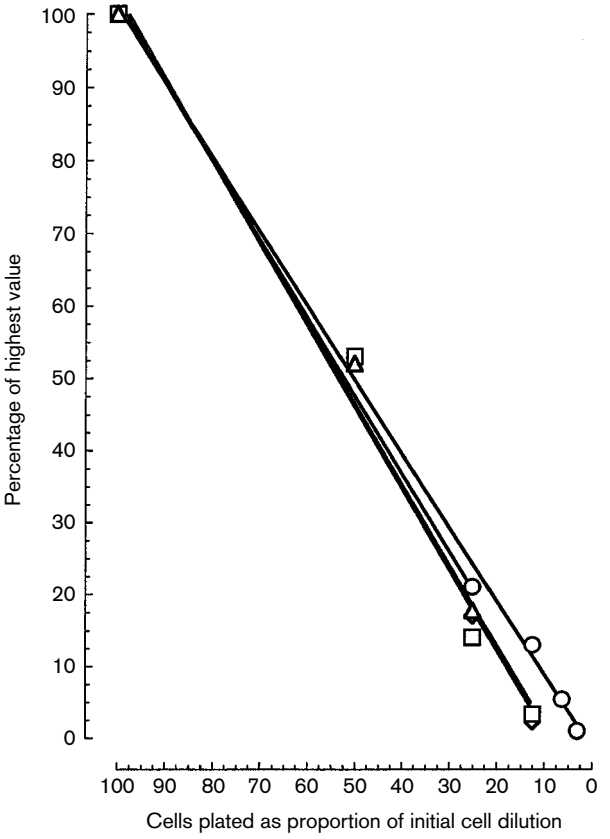

Figure 4. Linearity of the scintillation autofluorography assay following labeling with [^{35}S]methionine for 2 (△), 3 (□ or 4 (◇) h followed by treatment with salicylic acid. ○, Cell count.

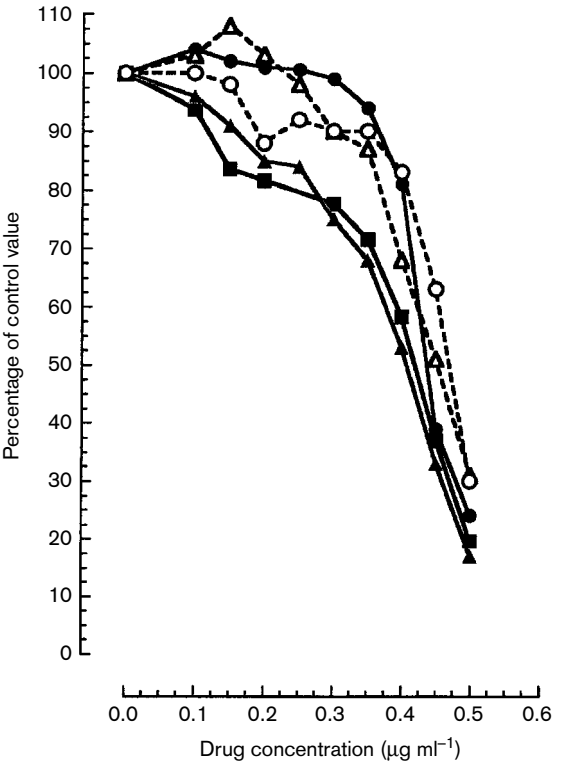

Figure 5. Comparison of endpoints in cultures of U251MG cells treated with 5-fluorouracil. ○, Scintillation autofluorgraphy (sodium salycilate). △, Scintillation autofluorgraphy (toluene-based scintillation fluid). ▲, Cell counting. ●, Scintillation spectrometry (scintillation counter 1). ■, Scintillation spectrometry (scintillation counter 2).

uptake) were negative, indicating that scintillation autofluorography is slightly less accurate than cell counting at detecting small numbers of cells. The relationship between isotope concentration and peak height was also linear over a concentration range of 0.4–2.0 μCi/ml of [^{35}S]methionine (data not shown).

Comparison of endpoints in a chemosensitivity assay

Four methods of determining the endpoint of a chemosensitivity assay were compared. Isotope uptake was measured in three ways: scintillation autofluorography using toluene-based scintillation fluid or sodium salicylate and by scintillation spectrometry. These methods were compared with cell counting. Results were calculated as a percentage of control values as determined by peak height measurement, scintillation counter results or cell counts and plotted as a dose–response curve (Figure 5). All four methods of assessing drug sensitivity correlated well, the range of apparent ID_{50} being 0.4–0.47 μg/ml. At 0.5 μg/ml, the highest drug dose used, the four methods assessed viability to be between 17 and 31%.

Discussion

Despite the introduction of non-isotopic methods to determine cell number like the MTT assay, isotopic uptake as a measure of cell number remains a popular and widespread methodology. These types of assay are in routine use for mass screening of drugs for potential activity in a variety of different types of tumor as well as individualized chemosensitivity testing. However, in order to do this safely and with minimal costs, the use of microtitration plates and inexpensive reagents is necessary.

Since the original description of a method to detect isotope uptake using a scintillation autofluorographic method,[1] this approach has been successfully used to demonstrate a relationship between *in vitro* chemosensitivity to a variety of cytotoxic drugs and relapse-free interval in patients with malignant astrocytoma.[10]

A major advantage of this method is that it provides a permanent record of each microtitration plates used, which is extremely useful for long-term quality control purposes. This scintillation autofluorographic method shows considerable promise as an efficient and accurate method for detecting radioisotope uptake in multiple well plates.

In the present paper, we describe a novel modification of this method which shows a linear relationship between absorbance and cell number, and shows close agreement with several other methodologies used to determine residual cell number following drug treatment. A major advantage of this technique is the relative non-toxicity of sodium salicylate which is in marked contrast to the organic solvent-based scintillation fluid. Toluene can be absorbed by inhalation and by skin contact, and whilst low-level exposure produces a wide range of toxicity's including irritation of the upper respiratory tract, chronic exposure has been linked with hepatotoxicity and nephrotoxicity. Xylene is no less toxic and causes a number of CNS effects following acute overexposure by inhalation, and eye and skin irritation following direct contact. In addition, the use of flammable chemicals like toluene and xylene which are not miscible with water provides a number of technical challenges in the laboratory which need to be addressed. Disposal of plates after the completion of the assay and any waste scintillation fluid may be a problem. This is not a result of the small amount of short-lived radioisotope which is present on each plate but because of the residues of organic solvent-based scintillation fluid.

The adoption of sodium salicylate, although not wholly without toxicity,[2] provides a number of distinct advantages. There are few problems with subsequent disposal of either plates or waste solutions of sodium salicylate. Similarly, the structural rigidity of the microtitration plate is not compromised by the use of methanol-based solutions of sodium salicylate. It is possible that this relatively non-destructive methodology could allow subsequently investigations to be carried out either before or after assessment of isotope uptake on the fixed cells these might include phenotypic characterisation of the target cells using immunochemical methods or changes in DNA or RNA levels using *in situ* hybridization techniques.

Comparative studies have shown that the typical peak height of wells treated with the toluene-based scintillation fluid used in this study were about 30% higher than wells treated with sodium salicylate (data not shown), but we have not carried out a systematic comparative studies of a wide range of different formulations of scintillation fluid with different concentration of fluors.

In summary, sodium salicylate provides a rapid and technically simple way to determine [^{35}S]methionine uptake in cells cultured from human malignant astrocytoma. It is less toxic and technically simpler to handle than organic-solvent based scintillation fluid, but provides identical data.

Acknowledgments

The authors are extremely grateful to Dr R Ian Freshney formerly of the University Department of Oncology, University of Glasgow, for his initial description of the scintillation autofluorography assay in 96-well microtitration plates and his continued interest in this assay.

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(Received 20 January 2000; accepted 1 February 2000)