A New Quantitative Film Autoradiographic Method of Quantifying mRNA Transcripts for In Situ Hybridization

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SUMMARY We developed and tested a novel quantitative method for the quantification of film autoradiographs, involving a mathematical model and a dot-blot-based membrane standard scale. The exponential model introduced here, \( ROD = p_1 \cdot (1 - \exp(p_2 \cdot x)) \), appropriately \((r^2 > 0.999)\), describes the relation between relative optical density (ROD) and radioactivity \((x)\) in the range between 0 and 240 gray scale values (using a 256-gray scale level digitizer). By means of this model, standard curves with distinct quenching properties can be exactly interconverted, permitting the tissue-equivalent calibration of different standard scales. The membrane standard scale employed here has several advantages, including the flexible radioactivity range, the facile and rapid preparation technique, and the compact size. The feasibility of the quantification procedure is exemplified by the comparative quantification of multiple calmodulin mRNAs in the rat brain by in situ hybridization with \([35S]\)-cRNA probes. The procedure for quantification provides a significant improvement in that the direct and exact comparison of radiolabeled species, even from different experiments, can be reliably performed. Further, the procedure can be adapted to the quantification of autoradiographs produced by other methods.

KEY WORDS quantitative autoradiography, in situ hybridization, image analysis, computer-assisted microdensitometry, mathematical model, calibration procedure, exponential function, \([35S]\) standards, calmodulin mRNAs, Hyperfilm-MAX

Autoradiography is a powerful method for revealing the spatial distribution of radiolabeled substances in biological materials. The choices for the high-resolution quantitative tracing of radioactivity in biological specimens are the grain counting of emulsion-coated sections (Lucas et al. 1994; Jonker et al. 1997), film densitometry (Ehn and Larsson 1979; Unnerstall et al. 1982; Kuhar and Unnerstall 1985; Baskin and Stahl 1993), and the storage phosphor screen technology (Sonoda et al. 1983; Amemiya and Miyahara 1988; Ito et al. 1995). Phosphor screens exhibit several advantages (e.g., a five order linear dynamic range, high sensitivity, and a short exposure time), but their application to tissue sections is still restricted by their poor maximal resolution. Therefore, traditional silver halide-based imaging methods are widely used, because even film autoradiographs offer a much higher spatial resolution and microdensitometry is still a straightforward, relatively rapid, and reliable means of quantification at considerably lower cost (as compared with the sophisticated and expensive phosphor scanners). Here, only the issues relevant to the quantification of film-based autoradiography for in situ hybridization involving \([35S]\)-labeling are discussed, but the method described below can readily be adapted to other autoradiographic systems also.

Although the fundamentals of the quantitative analysis of film autoradiographs have already been described (Ehn and Larsson 1979; Unnerstall et al. 1982; Kuhar and Unnerstall 1985; Davenport et al. 1988; Baskin and Stahl 1993), qualitative or semiquantitative investigations relating to the distribution of receptor ligands or hybridization probes are still quite common (Matsuoka et al. 1992; Gulya et al. 1993; Sola et al. 1996). The main problem with the quantification of autoradiographs is that the degree of darkness (gray level or grayness, usually expressed in optical density, OD) of film images is not linearly related to the radioactivity present in the specimen (Ehn and Larsson 1979; Unnerstall et al. 1982; Kuhar and Unnerstall 1985). In general, the relationship between the OD and radioactivity is such that the OD first increases...
slowly, but then more quickly at relatively low concentra-
tions of the radioisotope, and the slope of the curve sub-
sequently decreases at higher levels of radio-
activity as the film becomes saturated, and the OD fi-
nally reaches a plateau. Although the shape of the curve
is determined primarily by the nature of the photo-
graphic emulsion and the radionuclide utilized, it also
depends on other factors, such as the exposure
time, the details of the film processing, and the instru-
ment used for analysis.

The quantification of a radiolabel bound to experi-
mental tissue sections is performed by co-exposing a
series of calibrated radioactive standards. The aim of
using standards is to relate the signal units (here the
OD) accurately to calibrated units of radioactivity and
then, on the basis of this relationship, to determine the
quantities of radiolabel corresponding to the observed
signals of experimental samples. The two currently
used radioactive standard scales are the tissue paste-
and plastic-based ones. Tissue paste standards are
prepared by the investigator by mixing increasing
amounts of the radioisotope to be quantified with tis-
sue paste (Unnerstall et al. 1982; Davenport et al.
1988). Because the paste is derived from the tissue un-
der investigation, the quenching of radiation due to
variations in the media and the thickness and mount-
ing of the specimens is essentially the same in both the
standards and the tissue sections. In general, however,
the use of tissue paste standards involves a number of
technical disadvantages: complicated preparation, con-
tamination of the cryostat with radioactivity, a con-
siderable consumption of material, difficulties in re-
producing uniform radioactivities in subsequent scales,
and the necessity for frequent preparation of $^{32}$P-
, $^{33}$P-, $^{35}$S-, and $^{125}$I-labeled standards because of the
short half-lives of these radionuclides. Alternatively,
plastic standard scales are utilized, prepared from
poly(methyl methacrylate), containing increasing quanti-
ties of radioisotope. $^3$H, $^{14}$C, and $^{125}$I scales are
available commercially (Amersham, Arlington He-
ights, IL; American Radiolabeled Chemicals, St Louis, M O)
and possess quenching properties similar to those of
tissues. For proper quantification, plastic scales must
always be calibrated in terms of tissue-equivalent ra-
dioactivity (Davenport et al. 1988; M iller et al. 1988;
M iller 1991; Baskin and Stahl 1993).

With an adequate number of data points on the tis-
sue paste scale, the sensitometric curve of the film can
be determined. In the absence of an explicit theory de-
scribing the complex relationship between OD and ra-
dioactivity, third-order (Sokoloff et al. 1977; Gooche
et al. 1980) and fourth-order (Pan et al. 1983; Baskin
and Stahl 1993) polynomial, power (Unnerstall et al.
1982), or logistic functions (M iller et al. 1988) have
been utilized for approximation and interpolation of
the autoradiographic data of the standard scales. The
application of the above-mentioned functions is based
merely on empirical observations, with the intention
of finding the best fit (Baskin and Wimpy 1989). When
convenient plastic standards are calibrated to
tissue-equivalent radioactivity, calibration is performed
via such functions (e.g., polynomial functions; Baskin
and Stahl 1993). Because of the empirical nature of
the approach, the parameters of these functions can-
not be associated with any real meaning. O wing to the
approximation of the best fit, considerable variations
in the parameter values may be observed between ex-
periments. Therefore, the use of predetermined pa-
rameter values for quantification in subsequent studies
could lead to uncertain results, reducing the reproduc-
ibility and comparability of these procedures. A math-
ematical model based on an explicit theory may over-
come these problems and would facilitate a more
accurate analysis of film autoradiographs.

The study reported here presents a new quantita-
tive autoradiographic method which involves a novel
mathematical model of film autoradiography and a
membrane standard scale. The meanings of the pa-
rameters in the model are clear and can be associated
with the experimental conditions. This model can be
used for the approximation and interpolation of data
points on different standard scales. In addition, their
calibration to tissue-equivalent radioactivity can be
performed by means of a simple mathematical opera-
tion. A dot-blot-based $^{35}$S membrane standard scale,
its calibration to $^{35}$S brain paste standards (by means
of our model), and its subsequent use for the quantifi-
cation of in situ hybridization involving $^{35}$S-cRNA
labeling are also shown. The membrane scale, alterna-
tive to tissue paste or plastic standards, has several
advantages and can easily be adapted to other radiola-
beling systems. The use of our quantification method
allows accurate determination of the radiolabel in
terms of absolute units (e.g., mRNA copy numbers).
Moreover, even the quantitative evaluation of differ-
ent experiments can be reliably performed, which
therefore permits a direct comparison concerning the
amounts of labeled molecules. To demonstrate the
merits of our method, an example of the comparative
determination of the distribution of the mRNA species
corresponding to the three bona fide calmodulin (CaM)
genes in rat brain is presented.

Materials and Methods

Derivation of the Mathematical Model

The fundamental feature of film autoradiography is that,
during exposure, the absorption of radiation by silver halide
crystals in the photoemulsion reduces silver ions to silver at-
omas. In the developer, additional reduction of silver ions
takes place preferentially where silver atoms reduced by ra-
diation are already present, finally making the exposed area
gray. Therefore, the degree of grayness is determined by the quantity of silver ions reduced during the exposure. The mathematical model reported here describes how the quantity of silver atoms depends on the intensity and duration of the exposing radiation.

Let us expose an autoradiographic film to a homogeneous radiation source (all the following statements correspond to the exposed film area) and assume that the decrease in the concentration of silver ions in unit time at moment $t$ is proportional to the concentration of silver ions and to the intensity of the radiation. Let $E(t)$ denote the concentration of silver ions at moment $t$ of the exposure. In the time interval $[t, t + \Delta t]$, for small values of $\Delta t$, we then get

$$E(t + \Delta t) - E(t) = -C\Delta t$$

where $x$ is the intensity of radiation (cpm/mm$^2$; here, for simplicity, it is considered to be constant; the general case where radioactivity $x$ decreases in time will be considered below) and $C$ is a constant depending only on the film and the conditions of the experiment. Dividing by $\Delta t$ and taking the limit as $\Delta t$ tends to zero, we obtain the differential equation

$$\frac{dE(t)}{dt} = -C x E(t)$$

(2)

Dividing by $E(t)$ and integrating over the interval $[0, t']$ (where $t'$ is the time of exposure), we get

$$\ln E(t') - \ln E(0) = -C t' x$$

Raising to the power of the natural base leads to the formula

$$E(t') = E(0) \exp[-C t' x]$$

(3)

Therefore, if $C$ denotes the molar concentration of atomic silver at the end of the exposure, we have

$$C = E(0) - E(t') = E(0)(1 - \exp[-C t' x])$$

As $C$ cannot be measured directly, after the film has been developed, the degree of grayness (presumed to be homogeneous) as a function of light transmission is determined instead by microdensitometry. Grayness is characterized by its opacity ($O$; $O = L/L_0$, where $L$ is the amount of light incident on the film and $L_0$ is the amount of light transmitted). According to the Beer–Lambert law, $O = 10^{-C}$ ($C$ is a constant). To linearize the function between grayness and $C$, the OD as a new measure of grayness is introduced as

$$OD = \log_{10}(O) = c_1 C$$

(4)

When a 256-gray level digitizer is used, the grayness is expressed as a gray value (GV) on the linear scale between 0 (brightest) and 255 (darkest). The GV can be transformed into an OD-like value, the relative optical density (ROD; Baskin and Stahl 1993):

$$ROD = \log_{10} \frac{255}{255 - GV}$$

(5)

The ROD units display the same log-reciprocal relationship to light transmittance as the true OD and are reported to be closely correlated with the true OD values (in the OD range 0.05–2.4, $r^2 = 0.997$; Baskin and Stahl 1993). For practical purposes, except at the very light and very dark ends, ROD can be utilized instead of OD, and thus

$$ROD = c_2 C = c_2 E(0)(1 - \exp[-ct' x]) = p_1(1 - \exp[-p_2 x])$$

(6)

where $c_2$ is a constant. Parameter $p_1 = C_2 E(0)$ depends on the film [mostly on the initial concentration $E(0)$ of silver ions] and the conditions of film development; parameter $p_2 = ct'$ depends on the radioisotope, the film, the exposure time, and other experimental conditions.

In brief, we use nonlinear regression with the function

$$ROD = p_1(1 - \exp[-p_2 x])$$

(7)

for the approximation, interpolation, and calibration of autoradiographic data.

Generalization of the Mathematical Model

When the exposure time is not negligible relative to the half-life ($T_{1/2}$) of the radioisotope, the radioactivity $x$ varies considerably according to $x(t) = x(0) \exp[-\lambda t]$, where $\lambda$ denotes the radioactive decay parameter ($\lambda = \ln2/T_{1/2}$). If the film exposure starts at $t = 0$ and $x(0)$ denotes the activity at the beginning of the exposure, then formula (1) takes the form

$$E(t + \Delta t) - E(t) = -C E(t) \int_t^{t + \Delta t} x(s) \exp[-\lambda s] ds$$

(1*)

By means of operations identical to those performed in connection with formulas (1)–(4), from formula (1*) we get

$$ROD = p_1(1 - \exp[-p_2 x])$$

(4*)

where $p_1 = c_2 E(0)$ and $p_2 = ct'$, as before.

Formula (4*) allows determination of the sensitivity curves under general conditions, even when the exposure times are different. However, it should be emphasized that function (4) can be used to establish the relation between ROD and radioactivity, even if the duration of the exposure is comparable to $T_{1/2}$, provided that all the samples and standards are exposed for the same length of time. Since the exposure time was kept fixed (36 hr) in this study, formula (4) was applied for regression analysis.

Calibration Procedure of a Membrane Standard Scale

for Tissue-equivalent Radioactivity Using a Mathematical Transformation

Let us first determine the relation between the standard curves of tissue paste (b) and membrane (m) standard scales. Let us co-expose standards to and develop the film, evaluate the autoradiographic data, and apply regression analysis with formula (4). As parameter $p_1$ in formula (4) is a characteristic of the film and developing conditions, thus independent of quenching in different standards, the corresponding parameters for both scales are equal: $p_1^b = p_1^m$. Furthermore, because the two data sets are obtained under identical conditions, all factors except the different extents of quenching are equal in both parameter $p_2^b$ and $p_2^m$. Thus, the relation
between the different quenching factors of the standards can readily be determined by introducing the transformation quotient γ as follows:

\[ \gamma = \frac{p_2}{p_2 - p_1} = \frac{c_1/t}{c_1/t - c_1/t} = \frac{c_1}{c_1} \]

Now, through use of the predetermined γ value (characteristic of a particular fixed set of experimental conditions), the regression function \( ROD = p_1(1 - \exp(-p_2\gamma x)) \) of each of the subsequent membrane standard scales (z), processed with the fixed experimental conditions, can be simply transformed to that of the corresponding tissue paste scale by setting \( p_2 = \gamma p_2 \), i.e., \( ROD = p_1(1 - \exp(-\gamma p_2 x)) \). An example for the calibration procedure is provided in the Results section.

Experimental Animals and Tissue Preparation

Male Sprague-Dawley rats (200–250 g) maintained under standard housing conditions were decapitated and the brains were quickly removed for either brain paste preparation or in situ hybridization. All animal experiments were carried out in strict compliance with the European Communities Council Directive (86/609/EEC) regarding the care and use of laboratory animals for experimental procedures.

Twelve brain paste standards with halving radioactivity levels were prepared as described by Davenport et al. (1988). Briefly, six whole brains were rinsed to remove blood, blotted dry, and homogenized at 4°C. Known amounts of [35S]-labeled tissue paste in 2-ml microcentrifuge tubes. After vortexing for 10 min to ensure the homogeneous distribution of the radiolabel, tissue pastes were frozen at −70°C until further processing.

For in situ hybridization, brains were embedded in Cryomatrix cryo-embedding medium (Shandon Scientific, Pittsburgh, PA). Cryostat sections of the same thickness as that of the experimental brain sections (15 μm) were cut, thaw-mounted onto Cr-Al-gelatin-coated glass slides and allowed to dry. At intervals during the cutting process, representative sections were collected and their radioactivities were determined by liquid scintillation counting. The resulting radioactivity scale covered the range −2.2–5.28 × 10^6 cpm/mm² at intervals of approximately halving activity levels.

For in situ hybridization, brains were embedded in Cryomatrix and frozen immediately at −70°C. Serial coronal cryostat sections (15 μm) from selected brain areas were cut and thaw-mounted onto CryoAl-gelatin-coated glass slides. Sections were air-dried and stored at −70°C until further processing.

In Situ Hybridization

The protocol for hybridization with [35S]-cRNA probe was that of Gulya et al. (1993). Briefly, coronal cryostat brain sections were fixed for 5 min in 2 × SSC (0.3 M sodium chloride and 0.03 M sodium citrate, pH 7.0) containing 4% formaldehyde, washed twice in 2 × SSC for 1 min, then rinsed in 0.1 M triethanolamine containing 0.25% acetic anhydride for 5 min at room temperature (RT).
were dehydrated, air-dried, and hybridized in 50 µl hybridization solution [50% formamide, 6 × SSPE (0.9 M sodium chloride, 0.06 M sodium phosphate, and 0.006 M EDTA, pH 7.4), 5 × Denhardt’s reagent, 10% dextran sulfate, 0.5% SDS, 50 mM DTT, 100 µg/ml salmon sperm DNA, and 50 µg/ml yeast tRNA] containing 2.5 × 10^6 cpm cRNA probe/section. Hybridization was performed under Parafilm coverslips in a humidified chamber at 50°C for 20 hr. The sections were rinsed in 2 × SSC/50% formamide at RT for 5 min, twice in 2 × SSC/50% formamide at 50°C for 10 min, and in 2 × SSC at RT for 5 min. The sections were then incubated in 1 × TE (0.01 M Tris and 0.001 M EDTA, pH 8.0) buffer containing 0.5 M NaCl and 6 × 10^5 RNase A at 37°C for 30 min, and rinsed twice in 2 × SSC/50% formamide at 50°C for 10 min and in 2 × SSC at 50°C for 10 min. Sections were dehydrated, air-dried, and processed for autoradiography. Some tissue sections were counterstained with toluidine blue.

**Autoradiography**

Tissue sections and membrane standards were apposed to Hyperfilm-βmax autoradiographic film (Amersham, Arlington Heights, IL) at 4C for 36 hr. Films were developed in Kodak D19 developer (Eastman Kodak; Rochester, NY) at 19°C for 3.5 min and fixed in Kodak Fixer at 19°C for 10 min.

**Densitometry**

 Autoradiographic data were quantified by computer-assisted microdensitometry. The image analysis system consisted of a high-resolution, 24-bit flatted color scanner (Mikrotec IH HR; Mikrotec International, Taiwan, ROC) attached to a Power Macintosh 8100/80 AV. Video images of the autoradiographs were captured at 600 × 600 dpi resolution (pixel size ~42 µm) and analyzed by the public domain computer program NIH Image 1.59 (Rasband 1997). GV's between 0 and 255 were assigned to the grayness of certain selections of autoradiographic images (homogeneous grayness was assumed for the entire selection), the background (less than 20 GV) was subtracted, and the corresponding ROD's were determined. The relationship between the GV’s of the tissue paste standards and those of the brain sections was established on the assumption that identical concentrations of radioactivity in the standards and in the brain sections resulted in identical GV's in their autoradiographs (Kuhar and Unnerstall 1985; Davenport et al. 1988).

The autoradiographic images of both tissue paste and membrane standards were delineated on the computer screen, and their areas and the corresponding GV's were determined. Data were corrected for film background and expressed in ROD, and averages of three or four measurements were calculated. Radioactivity/pixel ± SD vs ROD ± SD was plotted and nonlinear regression analysis with function (4) was applied (Statgraphics 6.0; Statistical Graphics Corp. and Manugistic Inc., Rockville, M D). Membrane standards were calibrated to tissue paste standards and their activity was calculated in tissue-equivalent radioactivity. In situ hybridization autoradiographs were quantified by use of calibrated membrane scales. Film background-corrected ROD's (nonspecific hybridization was indistinguishable from the film background) of brain areas anatomically defined according to toluidine blue-counterstained sections (Paxinos and Watson 1986) were determined. Duplicate measurements were performed for every brain area analyzed in five animals. Radioactivity/pixel values corresponding to ROD measurements on defined brain areas were calculated by utilizing the interpolation of the co-exposed calibrated membrane standard scales. Messenger RNA contents of different brain areas were estimated via the formula

\[
\text{RNA copy no. / mm}^3 = \frac{\text{radioactivity (cpm/pixel)}}{\text{probe specific activity (cpm/mol) × Avogadro no. × f}}
\]

where the Avogadro no. = 6.0225 × 10^23 and f = 37792.9 is a correction factor to scale up a pixel volume (42 µ × 42 µ × 15 µ) to 1 mm^3. Final results were expressed in mRNA copy number ± SD.

**Results**

**Calibration of the 35S Membrane Scale to Tissue-equivalent Radioactivity**

Typical autoradiographic images of a series of radioactive brain paste and membrane standards are shown in Figure 1. RODs and areas corresponding to each member of the standards were determined, and regression analysis with function (4) was applied to both series. For both scales, values of r^2 = 0.999 were observed in the ROD range 0–0.85 (0–240 total GV; Figure 2). Data points corresponding to this range were used to determine parameter values: p1 = 0.8657 and p2m = 3.0252 for the membrane scale, and p1 = 0.8590 and p2b = 5.0702 for the brain paste scale. In support of the model, the p1 values differed from each other by less than 1%. Transformation quotient γ = p2b/p2m = 1.676 indicates that the absorption of β-emission was higher in the membrane than in the brain paste. The determination of γ was repeated with different pairs of standard scales, and almost identical values were obtained.

**CaM In Situ Hybridization Autoradiography**

To demonstrate the method, we performed a comparative determination of the distribution of multiple CaM mRNA species in the hypothalamic and adjacent areas at the level of bregma–1.3 mm in the rat brain. Coronal brain sections hybridized with the CaM I, CaM II, and CaM III cRNA probes were co-exposed with membrane standard scales (18 films with their own scales were used in this study). Regression parameters (p1m and p2m) corresponding to membrane standard curves were determined for each scale (r^2 > 0.9995), p2m values were transformed according to p2b = γp2m (b brain paste) into tissue-equivalent form, and the resulting equations, ROD = p1m(1 − exp[−γp2mx]), were utilized for the interpolation and
radioactivity of the resulting scale covered the range $\sim 2.2-5.28 \times 10^3$ cpm/mm² at intervals of approximately halving activity levels. (B) Halving dilutions of a radiolabeled and size exclusion chromatography-purified cRNA probe were prepared. Radioactivity was measured in a liquid scintillation counter. Probe dilutions (0.5–1.64 $\times 10^4$ cpm/mm²) were dot-blotted onto a nylon membrane and fixed by UV crosslinking. Membranes and tissue paste sections were dried and co-exposed to Hyperfilm-Max for 36 hr at 4°C. Bar = 10 mm.

approximation of radioactivity in defined areas of the co-exposed brain sections. The correctness of the method is demonstrated by the fact that the parameter values of the 18 standard scales exhibited low variance: $p_{1m} = 0.8528 \pm 0.0188$ (mean ± SD) and $p_{2m} = 3.3414 \pm 0.2227$ (mean ± SD). The autoradiographic images of the brain sections revealed a widespread and differential localization of CaM mRNAs (Figure 3). No measurable images were observed when the sense probe was hybridized to tissue sections (data not shown). Table 1 lists the determined CaM mRNA copy numbers of selected brain areas at the level of bregma–1.3 mm.

Summary of the Quantitative Method
1. Calibration of membrane standards to tissue-equivalent radioactivity
   1.1. Preparation of the radiolabeled tissue paste standard scale from the tissue under investigation
   1.2. Preparation of the membrane standard scale (the final radioactivity range should be in the same range for both scales)
   1.3. Co-exposure of the scales to film for the time appropriate for the experimental tissue samples
   1.4. Development of the film, measurement of the GVs and areas corresponding to the autoradiographic images, and calculation of the RODs and radioactivities for each member of the standards
   1.5. Performance of nonlinear regression analysis with function (4) for both scales, determination of $p_{1m}$, $p_{1b}$, $p_{2m}$, and $p_{2b}$ values (m, membrane; b, tissue paste) and calculation of $\gamma = p_{2b}/p_{2m}$.
2. Quantification of radiolabel corresponding to tissue sections
   2.1. Preparation of the membrane standard scales
   2.2. Co-exposure of the membrane standards and radiolabeled experimental tissue sections under the same conditions as in 1.3.
   2.3. Development of the films, evaluation of the autoradiographic data of the membrane standard scales and tissue sections as in 1.4. (except that no area measurement is necessary for brain areas)
   2.4. Performance of regression analysis with function (4) for the standard scales, determination of $p_{1m}$ and $p_{2m}$ values and calculation of corresponding $p_{2b} = \gamma p_{2m}$ with the value $\gamma$ determined in 1.5
   2.5. Use of the equation $ROD = p_{1m}(1 - \exp[-\gamma p_{2m}])$ to reveal the amounts of radiolabel corresponding to tissue sections.

Discussion
The response of the film to radioactive exposure was found to be similar to that reported by other authors (Unnerstall et al. 1982; Kuhar and Unnerstall 1985;
Davenport et al. 1988; Ito et al. 1995). For approximation, interpolation and calibration, in contrast with previous studies (Sokoloff et al. 1977; Unnerstall et al. 1982; Pan et al. 1983; Miller et al. 1988), a novel mathematical approach was introduced. The exponential model developed in this study \( ROD = p_0(1 - \exp[-p_2(x)]) \) utilizes two parameters and describes the \( ROD \) vs radioactivity relationship appropriately \( (r^2=0.999) \) between 0 and 240 (out of 255) GVs with Hyperfilm-\( \beta \)max. For higher GVs, regression analyses result in poorer fits \( (r^2 = 0.992) \). The first parameter \( (p_0) \) is determined by the autoradiographic film and developing conditions, which were kept fixed during our experiments. The second parameter \( (p_2) \) depends on the experimental conditions, which were kept fixed, but also involves the quenching of radioactivity which, in contrast, varies with the different tissue sections and standard scales. The main novel features of this model are (a) the clear theoretical basis, yielding parameter values with low variance even when different experiments are compared, and thus more accurate quantification, and (b) the determination of the transformation quotient \( \gamma \) for a particular experimental set and standard scales makes it possible to perform exact transformations of sensiometric curves and thereby accurately calibrate different standard scales to tissue-equivalent radioactivity.

Earlier attempts at tissue-equivalent calibration (e.g., Baskin and Wimpy 1989) were achieved with empirically selected mathematical functions in which, according to the approximation of the best fit, considerable variations in the parameter values could be observed in different experiments. Therefore, the use of those functions, especially when regression parameters were determined in one experiment and then used in subsequent quantification procedures, reduced the reproducibility and the comparability of the results. However, when not only relative changes in one particular experiment but the evaluation of extensive series of data from several experiments involving multiple labeled species are concerned, the application of our model is more advantageous.

A dot-blot-based \( {}^{35} \)S membrane standard scale was also introduced in this study. The preparation of this standard scale is fast and simple. Series of dilutions are prepared from the same probe as used for hybridization, dot-blotted, and fixed to a nylon membrane and then dried. The entire procedure takes less than an hour and requires little effort compared to the difficulties of preparing tissue paste standards (Davenport et al. 1988; Baskin and Stahl 1993). The radioactivity range of the membrane standard scale is highly reproducible but can be flexibly adapted to different experiments. It has a compact size comparable to that of the plastic scales but costs much less. Because of its favorable properties, the membrane standard scale seems a reasonable alternative to the existing standard scales. It is important to note, however, that size exclusion chromatography purification of the probe is obligatory, because only incorporated radionuclides are retained by the membrane. Since the incorporation of the radionuclides is 40–80% for cRNA synthesis, the use of a crude probe mixture will result in overestimation of the amount of blotted radioactivity. RNA contamination may cause a similar problem. Nevertheless, creating RNAase-free conditions does not in-

**Table 1** Quantitative determination of CaM mRNAs in rat brain

<table>
<thead>
<tr>
<th>Brain area</th>
<th>CaM I mRNA [copy no/mm³ ± SD (×10⁶)]</th>
<th>CaM II mRNA [copy no/mm³ ± SD (×10⁶)]</th>
<th>CaM III mRNA [copy no/mm³ ± SD (×10⁶)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piriform cortex</td>
<td>82.83 ± 1.40</td>
<td>85.31 ± 11.76</td>
<td>100.81 ± 8.53</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>5.83 ± 0.62</td>
<td>5.69 ± 0.71</td>
<td>10.44 ± 1.23</td>
</tr>
<tr>
<td>Caudate putamen</td>
<td>18.03 ± 2.17</td>
<td>24.59 ± 2.90</td>
<td>16.58 ± 2.44</td>
</tr>
<tr>
<td>Supraoptic n.</td>
<td>33.84 ± 3.00</td>
<td>18.80 ± 2.27</td>
<td>33.80 ± 2.95</td>
</tr>
<tr>
<td>Suprachiasmatic n.</td>
<td>28.71 ± 4.47</td>
<td>14.25 ± 1.09</td>
<td>18.28 ± 1.66</td>
</tr>
<tr>
<td>N. lat. olfactory tract</td>
<td>55.64 ± 8.26</td>
<td>64.08 ± 8.46</td>
<td>76.02 ± 9.49</td>
</tr>
<tr>
<td>Thalamus (total)</td>
<td>18.72 ± 4.40</td>
<td>19.00 ± 2.74</td>
<td>25.94 ± 1.98</td>
</tr>
<tr>
<td>Reticular thalamic n.</td>
<td>24.55 ± 2.68</td>
<td>13.56 ± 3.23</td>
<td>22.14 ± 3.23</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>3.95 ± 0.40</td>
<td>3.91 ± 0.99</td>
<td>5.37 ± 0.87</td>
</tr>
</tbody>
</table>

*Significant differences in copy numbers \( (p<0.01, \text{Student's two-tailed t-test}) \).
volve any extra steps, because in situ hybridization to mRNA is essentially performed in such an environment. In this study, the membrane was proved to retain 100% of the radiolabel when the conditions mentioned above were adhered to. Although the membrane scale described here was prepared from a [35S]-cRNA probe, the method might be adapted to oligo or DNA probes labeled with different radionuclides (e.g., 32P, 33P, or 3H).

To demonstrate the feasibility of our quantification procedure, the distribution of CaM mRNAs belonging to the three bona fide CaM genes was determined in certain hypothalamic and adjacent areas in the rat brain by in situ hybridization. Quantification was performed by use of the calibrated membrane standard scales. Our results indicate a widespread, area-specific and differential CaM gene expression in the rat brain.

To the best of our knowledge, this is the first copy number determination for the multiple CaM mRNA species. In general, these results are in agreement with the findings of other authors on rodent brains (Atsukawa et al. 1992; Gannon and McEwen 1994; Barrón et al. 1995; Sola et al. 1996). Because all of these studies are qualitative, their comparison with our results is quite cumbersome. Nevertheless, the relative proportions of signal levels in different areas are similar to those of our results. However, our method demonstrated further evidence for the differential expression in several brain areas, thus far not revealed (piriform cortex, globus pallidus, supraoptic and suprachiasmatic nuclei). The significant improvement achieved in this study is that the detected mRNA levels corresponding to the three distinct CaM genes can be compared directly and their real proportions can be determined quantitatively rather than in terms of relative values normalized to a distinguished area (Sola et al. 1996). It is essential to note, however, that our results indicate the copy numbers of the hybridized probe, which underestimate the true mRNA copy numbers for several reasons: degradation of mRNA may occur during the tissue preparation, fixation may not retain all mRNAs, and retained mRNAs may be in a conformation that is not favorable for stable hybridization, or are not actually accessible for probe hybridization. However, these factors should be similar for most or all of the different mRNA species.

To summarize, we have developed and tested a novel quantitative method with a new mathematical model and a membrane standard scale. This exponential model makes it possible to approximate, interpolate, and (on the basis of their different quenching properties) also differentiate, transform, and calibrate between autoradiographic images of specimens. This highly advantageous model has been applied to calibrate our dot-blot-based membrane standard scales to tissue paste standards and to quantify and compare the amounts of the multiple CaM mRNA species in the rat brain. The model, the calibration procedure, and the membrane scale can be used for the quantification of film autoradiographs of in situ hybridization or of other applications, such as receptor binding studies.

Acknowledgments

Supported by the Hungarian Science Foundation (OTKA No. T 22822 to KG and OTKA No. F 22658 to AP) and by the National Council on Technical Development (OMFB No. 97-20-M U-0028 to KG).

The skillful technical assistance of Ms Susan Ambrus and Ms Maria Kosztka is greatly appreciated.

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