

Appraisal of state-of-the-art

# Drug localization and targeting with receptor microscopic autoradiography

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## Abstract

This review is an argument in favor of better drug target identification. It presents the many merits and feasibilities of drug localization and target identification through the use of a suitable technique: receptor microautoradiography. Studies of drug targets and target bioavailability require methods with high resolution and sensitivity to gain information for understanding mechanisms of action, sound modeling, prediction of effects, and toxicity. For in vivo localization of drugs in tissues and cells, receptor microautoradiography was specifically designed to preserve both tissue structure and deposition of noncovalently bound diffusible compounds and to enable microscopic viewing, quantitative analysis, and characterization of target sites. This method and its applications are explained here. Pictorial and quantitative data are provided together with a discussion of identified targets that document the utility of receptor microautoradiography. For example, when applied to quantitative studies of vitamin D compounds, pharmacokinetic data of blood differed from those of target tissues and even among target tissues. Many of the target tissues discovered and characterized with receptor microautoradiography remained unrecognized with common ADME procedures, radioassay–HPLC, and whole-body autoradiography. For a visual overview of the multiple vitamin D targets, a *drug homunculus* has been composed. Such a drug or *target homunculus* may be created for any drug, dose, and time to aid in documenting and fingerprinting. Receptor microautoradiography also is a sensitive method. It can be used for the study of low-dose stimulatory actions of toxic substances to show relationships of receptor binding to dose-dependent reversal of effects, known as *hormesis*. In addition, a combination of *autoradiography and immunocytochemistry* with radiolabeled drug and antibodies to receptor or other cellular product permits further target characterization. In its own league, receptor microautoradiography provides unique information. Through greater detail and certainty, it can validate and complement less-sensitive approaches, decrease the failure rates of current ADMET predictions, and serve as a diagnostic tool and guide for biochemical, functional, and clinical follow-up in drug research and development. © 2004 Published by Elsevier Inc.

**Keywords:** ADME; Bioavailability; Drug homunculus; Drug localization; Hormesis; Methods; Pharmacokinetics; Prediction; Receptor microautoradiography; Rodents; Target tissue; Drug target validation; Toxicology; Transporters; Vitamin D; Whole-body autoradiography

## 1. Introduction

### 1.1. In vivo drug distribution

Knowledge about in vivo tissue and cellular distribution of drugs and their metabolites is important for understanding and predicting a drug's action and toxicity. Therefore, identification of in vivo target tissues and cells, their location and pharmacokinetics, are a worthy pursuit in

drug research and development (Koelle, 1969; Leake, 1969). Receptor microautoradiography with its documented high resolution and sensitivity can provide the necessary information.

The utility of common expedient low-resolution techniques, such as radioassay and whole-body autoradiography, needs to be critically assessed with respect to target identification. Limitations of these techniques—also in vitro incubation, high-throughput screening, in silico modeling, and noninvasive scanning—make it necessary to validate their results (Drews, 2000) through comparisons with results from high-resolution in vivo tissue and target distribution studies. For meaningful interpretation of data, comparative

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studies are indicated. Complementary information from approaches that provide high tissue and cellular *in vivo* resolution should be included.

A complete picture of the *in vivo* tissue distribution of a drug cannot be obtained through current ADME low-resolution procedures, neither alone nor combined with *in vitro* techniques or noninvasive scanning. Moreover, information about high-specificity–low-capacity tissue and cellular *in vivo* binding cannot be obtained through extrapolation. Kinetics of low-specificity–high-capacity binding nontarget sites, registered predominantly with radioassays and noninvasive scanning, differ from kinetics of high-specificity–low-capacity binding target sites. Different target cell populations may even have different target-specific binding characteristics and kinetics. Therefore, in drug research and development, individual target cell populations need to be considered by including microscopic resolution that allows identification of specific *in situ* cell types within each topographic context.

### 1.2. History

Attempts at tissue localization of bioactive substances, utilizing radiation signals, date back to 1930 (Lomholt, 1930), when a natural radioisotope was applied as a tracer. After reactors produced artificial radioisotopes, compounds could be labeled and new applications became possible. Autoradiography evolved as a histochemical method (Boyd, 1955; Hamilton, Soley, & Eichhorn, 1940) with various modifications of technique over time.

Microscopic autoradiography developed during the late 1940s and 1950s and was based on the use of tissues prepared by conventional liquid fixation and infiltration with embedding medium through sections apposed to radiation-sensitive film or coated with liquid nuclear emulsion. This approach yielded high cellular and subcellular resolution that resulted in numerous discoveries with radiolabeled precursor molecules for macromolecular synthesis of DNA, RNA, and proteins (Harbers, 1958; Schultze, 1969). Chemical bonding of the labeled compound and incorporation into large molecules, immobilized through denaturing fixatives, enabled retention and preservation of select *in vivo* tissue uptake. When the same type of method was applied to the localization of drugs, however, radiolabeled compound was translocated and lost during preparation of the autoradiogram. This is because drugs are nonincorporated, mostly weakly bound or unbound diffusible compounds. At sites of action, transitional binding to receptor protein mostly exists through weak ionic and van der Waals forces that can easily be broken during tissue preparation by fixation and dehydration fluids, embedding media, and coating with liquid emulsion.

It became apparent that localization of drugs in tissues at their authentic cellular and subcellular sites of binding and deposition would require special preparations and precautions (Feinendegen, 1968; Roth & Stumpf, 1969; Stumpf &

Roth, 1964; Ullberg, 1954). A microscopic technique had to be developed that excluded any steps that could cause translocation and loss of tissue constituents. Preserving tissue structure and, at the same time, retaining diffusible compounds at their *in vivo* sites was considered very difficult or even impossible (Levi, 1969). In early efforts that managed to preserve tissue structure and cellular resolution, radiolabeled molecules were lost from their original sites; and when the *in vivo* localization of the drug was maintained, preservation of the tissue structure and the desired resolution were lacking. Many modifications and “new” techniques were tried and recommended in the literature. A review of the failed techniques for the autoradiography of diffusible substances, while instructive, would be beyond the scope of this article. Most of the techniques recommended in the literature had to be abandoned. Claims that were made could not be confirmed. Confusion resulted and the utility of microautoradiography for drug research was questioned.

Because of these difficulties and the significant demands on attention to detail and skill that high resolution requires, investigators resorted to shortcuts. Sacrificing resolution and adopting easy sandwich procedures, researchers temporarily assembled tissue slices mounted on slides with sheet film or emulsion-coated slides for exposure and then separated them for treatment and evaluation. Whole-body autoradiography is a convenient apposition technique. Like other techniques with temporary assembly of radiation source and recording medium, whole-body autoradiography is limited in sensitivity and resolution. High resolution requires close and permanent contact between tissue and recording medium. While trying to arrive at a workable method for drug localization in tissues and cells (Roth & Stumpf, 1969; Stumpf, 1969), investigators abandoned their efforts.

The development of receptor microautoradiography was a long-awaited *breakthrough*. Along the arduous path, it was discovered that frozen sections can be cut at very low temperatures (Stumpf & Roth, 1965), that freeze-dried frozen sections can be used for histological studies without fixation and embedding (Stumpf & Roth, 1967), and that drugs and other diffusible compounds with high microscopic resolution can indeed be localized without translocation and loss (Stumpf & Roth, 1964, 1966). Since its inception, this method for the localization of hormones and drugs has enabled many discoveries and has given rise to many new concepts (Stumpf, 2003)—and yet, its full potential remains to be utilized.

### 1.3. Detection of drugs in target cell populations: sensitivity and resolution

Actions of drugs, xenobiotics and autacoids, mostly are understood through their relation to receptors, the quantity of molecules and receptors, and the duration of interaction. The precise number of molecules required for eliciting a specific action is unknown and may vary depending on the

drug. Estimates of receptor-bound molecules per steroid hormone target cell indicate a low number of a few thousand (Clark, Anderson, & Peck, 1973; Clark & Gorski, 1970; Clark, Stumpf, Sar, & DeLuca, 1987; Notides, 1970; Stumpf, Sar, Zuber, Soini, & Tuohimaa, 1981). There is evidence that the same compound will act differently at different target tissues, depending on affinity, amount and time-course of uptake and retention, and capacity for saturation (Keefer, 1981; Koike, Hayakawa, Kumaki, & Stumpf, 1998; Koike, Ichikawa, Nishii, & Stumpf, 1998).

Detectability of radiolabeled drug associated with the target is influenced by a number of factors, such as:

- receptor density and occupation,
- radiation type and energy of the label,
- specific activity of the labeled compound,
- resolution related to tissue structure, and
- sensitivity of the detection medium.

All these factors require consideration in the design of experiments and are discussed in detail elsewhere (Stumpf, 2003).

Enormous differences in detectability by different methodologies, in which the above factors play a role, have become apparent in comparative studies with radiolabeled vitamin D compounds. With whole-body autoradiography and radioassay, about 10 tissues were recognized to have radioactivity levels above that of blood. By contrast, with microautoradiography in more than 50 tissues, concentration of radioactivity was noted, and, through competition suppression, specific cell types could be characterized as genomic targets (Stumpf, 1995).

#### *1.4. Low-resolution whole-body autoradiography and radioassay*

Whole-body autoradiography provides expedient surveys and convenient pictorial documentation of gross distribution of radiolabeled drugs and metabolites that correlate with data from radioassay with excised tissues (Steinke et al., 2000). Whole-body autoradiography and radioassay are routine procedures in preclinical ADME studies at pharmaceutical companies. With both methods, detection of *in vivo* tissue distribution of radiolabeled compound is possible but restricted to regions with relatively high accumulation.

It is noteworthy that Ullberg, who first introduced whole-body autoradiography, recognized these shortcomings and tried to adapt Scotch tape-backed tissue sections for high tissue and cellular resolution (Hammarstroem, Appelgren, & Ullberg, 1965). This was apparently unsuccessful, and there were no follow-up experiments. Saran wrap-backed kidney sections (Wedeen, 1969) were also abandoned. An 'improved' whole-body method with thin sections and a new glue-covered plastic foil has been recommended for microscopic autoradiography (Kawamoto, 1990), but its use

for general tissue localization of drugs remains to be demonstrated.

Whole-body autoradiography (Som, Sacker, & Oster, 1995; Waddell & Marlowe, 1977) informs about high-capacity–low-affinity binding sites of radioactive compounds as related to dose and time, for which it can provide convenient overviews. In most cases, it is not possible to recognize specific target sites, that is, to distinguish sites of specific binding to receptors from sites of less-specific nontarget deposition. These limitations are related to technique and have several reasons, including ice crystal damage of tissue morphology during slow freezing of a whole animal, thick tissue sections of 20 to 50  $\mu\text{m}$ , high doses of labeled compound with low specific activity, a relatively large distance between tissue and detection medium, and the absence of competition experiments for identification target-related low-capacity binding sites. With whole-body autoradiography, the recognized distribution of radiolabeled compound is related mostly to contents in blood vessels; in cellular and extracellular compartments of metabolic organs, like liver, kidney, and adrenal; in excretory organs of the biliary, urinary, and gastrointestinal systems; in certain secretory glands; and to association with melanin.

Because specific sites of receptor binding frequently cannot be identified with whole-body autoradiography, it, like routine radioassay, is not suited for reliable target tissue identification and related *in vivo* receptor-drug pharmacokinetics. This deficiency considerably limits its diagnostic and predictive value for drug action and toxicity.

#### *1.5. High-resolution dry-mount and thaw-mount autoradiography*

The foundation for receptor microautoradiography was laid during the 1960s with the development of dry-mount and then thaw-mount autoradiography. These methods have been applied and perfected over several decades, resulting in what is now called receptor microautoradiography. During the development of this procedure, several important discoveries were made. It was learned that tissue could be frozen and frozen tissue could be sectioned in a manner in which cellular structure could be preserved for high light microscopic resolution (Stumpf & Roth, 1965, 1967) and subsequently even for electron microscopy (see Roth & Stumpf, 1969). Freeze-dried sections, previously thought applicable only to cellular biochemistry with the Lowry technique, had entered the realm of histology (Stumpf & Roth, 1964, 1966). Thin frozen freeze-dried tissue sections could be prepared, and their structure well preserved without fixation and without the use of embedding media, even be dry-mounted on photographic emulsion without any fluid treatment that was normally considered prerequisite for avoiding translocation and loss of unbound or weakly bound substances.

The ability to retain diffusible compounds and to demonstrate them within histological detail at their original sites *in vivo* was achieved with two diffusible compounds known to be localized at specific sites:  $^3\text{H}$ -estradiol in cells of the uterus and  $^3\text{H}$ -mesobilirubinogen in the biliary system of the liver (Stumpf & Roth, 1966); tested further with extracellular space indicators (Roth & Stumpf, 1969).

Only after successful testing and demonstration of their utility were the newly developed techniques recommended. The *dry-mount technique* was more pristine but more complicated. Its results served as a control for the less-involved, but less-assured thaw-mount technique. The *thaw-mount technique* excluded freeze-drying and dry-mounting of freeze-dried sections by attaching thin frozen sections to dried emulsion-coated slides through a brief pick-up and melting. Thaw-mounting was simpler and more expedient, but it introduced a liquid phase through melting. Melting of frozen sections remained a source of concern because of possible artifacts. This step in the procedure needed to be controlled and performed in a fashion to minimize or avoid artifacts. Accordingly, through multiple experiments, it was established that frozen sections had to be thin, i.e., 4  $\mu\text{m}$  or thinner. With thicker sections, as used in other autoradiographic procedures recommended in the literature (Baker, 1989), excess melting fluid and wet tissue could cause diffusion of labeled compound and loss of tissue structure, as well as interaction between tissue and nuclear emulsion followed by negative and positive chemography, altogether resulting in impaired resolution associated with various artifacts. Methods of microautoradiography that advocate the use of frozen sections above 5  $\mu\text{m}$  thickness, as recommended in the Appleton technique (Baker, 1989), should be considered unsafe and may produce inconsistent “results”, i.e., data mixed with artifacts.

Frozen section dry-mounting on photographic emulsion has been attempted and claimed by some investigators to be possible. In the author’s own experiments, when body heat and rise in temperature were carefully avoided during section pick-up, frequent loss of sections occurred during photographic processing. Adherence of frozen sections appeared to be linked to transitional melting and pressure, which was sometimes associated with refreezing and frequently with artifacts.

## 2. Receptor microautoradiography

### 2.1. Prerequisites

Receptor microautoradiography is not usually complicated, nor does it require a long list of materials. The main prerequisites for receptor microautoradiography are

- radiolabeled compound with high specific activity,
- nuclear emulsion,
- microtome cryostat,

- darkroom,
- liquid nitrogen tissue storage container(s), and
- microscope with image analysis facilities.

Basic knowledge in cell biology and pharmacology and careful attention to detail enhance success.

### 2.2. Experimental strategies

Microautoradiography, because of exposure times of weeks or months, may be applied selectively for specific compounds. Through careful planning and execution, the information gained is likely to be invaluable, providing leads for biochemical follow-up and functional and clinical pursuits that will accelerate discovery, evaluation, and introduction of new drugs. Experimental planning considerations are discussed in more detail elsewhere (Stumpf, 2003). Radiolabeled compounds can be administered in various ways as to mode and route of administration, dose, and time. The focus of study may variously emphasize routes of delivery and absorption, sites of metabolism and excretion, receptor binding with quantitative assessments related to time and dose, endocrine status, or pretreatment with competitor. After administration of radiolabeled compound to rodents (mice or rats are commonly used), tissue samples are excised and preserved by a special freezing technique.

With receptor microautoradiography, specific sites of tissue and cellular deposition of design drugs can be evaluated qualitatively and quantitatively, and data can be compared with each other. Computerized data can be used for correlative imaging, for creating a “drug homunculus” (Fig. 17) for overviews of target distribution and binding hierarchies, as well as for *in vivo* drug-receptor pharmacokinetics. Radiolabeled cells can be identified and characterized in the context of neighboring cell populations and topographic–functional relationships. Target cell populations can be further characterized as to their products and functions through a combined application of cellular autoradiography with immunocytochemistry or other histochemical procedures.

Pilot experiments are recommended. They can provide quick information for screening and effective design of focused follow-up experiments. Pilot studies may be performed with one or two animals per condition and with two dose levels, a low dose (physiologic or near-physiologic) and a high dose (3 to 5 times the low dose), as well as two or three time intervals, depending on the purpose of the study. Data from previous radioassays and biochemical studies may also be considered for the design of autoradiographic experiments and for comparisons of data. Tissues selected for excision should include both expected target as well as nontarget tissues for comparison and serendipity. Competition with excess unlabeled compound will be necessary for the characterization of localized radioactivity, i.e., the identification of high-specificity–low-capacity bind-

ing sites. Competition may be postponed as a follow-up experiment after evidence for target location has been obtained and suitable conditions of dose and time have been assessed.

### 2.3. Radiolabeled compounds

Radiolabeled compounds with high specific activity and purity are a sine qua non for receptor microautoradiography.

*Tritium-labeled compounds* are preferred and should be specifically labeled. General Wilzbach-type exchange-labeled compounds are not suitable, since the labeling is unstable and the achievable specific activity is low. Synthetic labeling should achieve a sufficiently high specific activity to allow detection of low-capacity–high-specificity target sites after application of a sufficiently low dose that does not obscure target uptake and retention by overloading secondary less-specific sites of binding and deposition. This complex situation, including relationships to specific tissue concentration of labeled compound and length of exposure time, is discussed in more detail elsewhere (Stumpf, 2003). With tritium-labeled steroids, target tissues can be detected with specific activities preferably above 50 Ci/mM. Compounds of high specific activity and multiple labeled sites are optimal. Autoradiolysis and purity need to be controlled.

<sup>14</sup>C-labeled compounds are less suitable for receptor-binding studies because they usually achieve a much lower specific activity in the mCi/mM range compared to tritium-labeled compounds in the Ci/mM range. As a result, low-capacity target sites with low specific tissue concentration are likely to remain undetected with <sup>14</sup>C-labeled compounds. In addition, <sup>14</sup>C-labeled compounds provide a lower autoradiographic resolution than <sup>3</sup>H- and <sup>125</sup>I-labeled compounds because of silver grain tracks due to high beta energy. <sup>14</sup>C creates curved tracks of several silver grains rather than singular spots of one or two silver grains, as is the case with tritium.

<sup>125</sup>I-labeled compounds are suitable (Stumpf, Morin, Ennis, Zielinski, & Hochberg, 1987). Because of the short half-life of <sup>125</sup>I, very high specific activities (2000 Ci/mM) are possible with related short exposure times. Because of the low energy of its Auger electrons, resolution with <sup>125</sup>I approaches that of tritium and is superior to that of <sup>14</sup>C. However, radiolabeling with <sup>125</sup>I and use for high-resolution autoradiography must take into account its short half-life with early decay and the large size of the iodine atom. When <sup>125</sup>I is attached to a small molecule, depending on position of the label and size of the molecule, chemical properties and binding affinities of the labeled compound to receptor may be altered. Appropriate tests are required to ascertain unaltered binding behavior.

Other radioisotopes have been used for autoradiography. Specific activity and resolution will have to be considered and may be limiting factors.

To recognize *receptor binding sites*, labeled drug should be applied in doses that are near-physiological or at the lower range of a pharmacological dose. Low-capacity–high-specificity receptor binding sites can, in this way, be visualized and distinguished from high-capacity–low-specificity sites. When high doses of compound with low specific activity are used, receptor sites of limited capacity may become saturated with a large proportion of unlabeled compound, thereby becoming unrecognizable in the autoradiograms because of weak radiation signals, and/or strong obscuring signals from adjacent high-capacity–low-specificity sites.

For *toxicological studies*, multiples of receptor-binding doses may be used and the results can be compared with those from low dose experiments. Experience with such comparisons is lacking in the literature, but can be gained using a sensitive method like the one described here. Important toxicological information and prediction may be derived from comparisons of different dose experiments, possibly providing clues to help explain phenomena such as ‘threshold’ and ‘hormesis’, i.e., reversals from low-dose stimulatory to high-dose inhibitory and toxic actions.

### 2.4. Laboratory and equipment

A laboratory for microautoradiography requires relatively little equipment:

- a lightproof darkroom for frozen sectioning and handling of emulsion-coated slides,
- a microtome cryostat with fiber optics cold light and a 15-W safelight with Kodak OC-filter or equivalent,
- a water bath and a drying rack for liquid emulsion coating of slides,
- Dewars and liquid nitrogen containers for tissue freezing and storage,
- a refrigerator and freezer for storage of nuclear emulsion and exposure of section-mounted slides in lightproof desiccator boxes,
- containers for developer, fixer, rinse, and stain,
- a light microscope for assessing section quality, orientation, and stain during sectioning, thaw-mounting, and after photographic processing,
- a photomicroscope with computer for the evaluation of developed autoradiograms, documentation, and quantitative image analysis.

### 2.5. Procedure

Table 1 summarizes the main procedural steps of receptor microautoradiography in a flow chart.

After administration of radiolabeled drug, the animal is sacrificed and organs or organ pieces are excised, positioned on tissue holders, and freeze-mounted by immersion in isopentane cooled by liquid nitrogen. The freeze-mounted

Table 1  
Receptor microautoradiography

Sequence of steps
<b>APPLICATION</b>
of radiolabeled compound with high specific activity (including competition)
↓
<b>EXCISION</b>
of multiple tissues and positioning on holders
↓
<b>FREEZE-MOUNTING</b>
on tissue holders
↓
<b>LIQUID NITROGEN STORAGE</b>
of mounted tissues
↓
<b>CRYO-SECTIONING</b>
of 4 μm (or less) sections
↓
<b>THAW-MOUNTING</b>
on emulsion-coated slides
↓
<b>PHOTOGRAPHIC EXPOSURE</b>
in desiccator boxes
↓
<b>PHOTOGRAPHIC PROCESSING</b>
↓
<b>STAINING with MBBF or MGP</b>
↓
<b>AIR-DRYING and COVER-SLIPPING</b>
↓
<b>EVALUATION</b>
qualitative and quantitative

Histological fixation, generally, is not necessary and therefore omitted. Only with combined autoradiography–immunocytochemistry is a brief histological fixation with 2–4% buffered paraformaldehyde recommended at the end of exposure before photographic processing. Common fixatives are known to cause artifacts. If fixation is used, controls are indicated. Single-step stains, MBBF=Methylene Blue–Basic Fuchsin or MGP=Methyl Green–Pyronin, are recommended. They are superior to the common H&E stain. For further detail, see Stumpf (2003).

specimens are kept in a liquid-nitrogen storage container until sectioning.

Four-μm sections are cut in a microtome-cryostat. The sections are then thaw-mounted on emulsion-coated slides, and the mounted slides are stored in lightproof desiccator boxes and placed in a freezer for exposure. The exposure times vary, depending on the specific activity of the labeled compound and the specific concentration of labeled compound in the tissue, as well as the desired density of developed silver grains. Because the concentration of labeled compound is unknown, exposure times cannot be calculated and they have to be determined empirically. Exposure times may amount to a few weeks or several months. Different exposure times can provide valuable information suitable for quantitative and qualitative evaluation. With short exposure times, individual silver grains can be counted at high magnification, suitable for quantitative evaluation. With long exposure times, silver grains are dense, clumped, and more easily visible at low magnifica-

tion, suitable for surveys. Because photographic exposure allows radiation signals to be stored and amplified over time, even small amounts of labeled compound may become detectable and can be related to cell and tissue structures in the developed and stained autoradiogram.

During tissue handling and preparation of autoradiograms, each step must be executed with care and precision. Because the method is highly sensitive, any mistake or modification of procedure may produce artifacts. Adequate controls are necessary.

## 2.6. Characterization of localized radioactivity

Characterization of localized radioactivity is important. It can be aided through information from receptor microautoradiography. For example, *specific distribution patterns* can be revealed for labeled compound to defined cell populations, such as, certain neurons in recognized regions of the brain or spinal cord (Stumpf & Grant, 1975), B-cells of pancreatic islets, kidney podocytes, and pituitary thyrotropes (review Stumpf, 1995). *Competition* is commonly applied. Selective suppression of target tissue uptake and binding of labeled compound can be observed through autoradiographic competition experiments with excess unlabeled compound or competitor. Results from *radioassay–HPLC* and *biochemical analyses* of topographically excised tissues may provide supportive evidence.

*Autoradiography combined with immunocytochemistry (Colocalization)* can further contribute to the characterization of target cells through simultaneous demonstration of radiolabeled progestin and antibodies to receptors in uterine tissues (Gasc, Ennis, Baulieu, & Stumpf, 1983), radiolabeled estrogen and antibodies to trophic hormones in the pituitary (Keefer, Stumpf, & Petrusz, 1976), peptide hormones and neurotransmitters in the brain (Stumpf & Grant, 1975), radiolabeled vitamin D compounds and antibodies to atrial natriuretic factor in cardiomyocytes (Bidmon, Gutkowska, Murakami, & Stumpf, 1991), tyrosine hydroxylase in adrenal medullary cells (Puchacz, Stumpf, Stachowiak, & Stachowiak, 1996), TSH in pituitary thyrotropes (Sar, Stumpf, & DeLuca, 1980), B-cells in endocrine pancreas (Clark, Stumpf, & Sar, 1981), and gastrin in stomach endocrine cells (Stumpf, Sar, O'Brien, & Morin, 1988).

## 2.7. Various applications

Numerous compounds have been localized and the localization has been characterized with receptor microautoradiography. These compounds, mostly labeled with  $^3\text{H}$  or  $^{125}\text{I}$ , include, e.g., sex and adrenal steroids (Stumpf & Sar, 1976), vitamin D (Stumpf, 1995, 2003), retinoic acid (Stumpf, Bidmon, & Murakami, 1991), glucose, and 2-deoxyglucose (Duncan & Stumpf, 1991). Drug delivery routes have been demonstrated, for instance, for blood–brain barrier-related entry of dexamethasone into the brain via cerebrospinal fluid

(Rees, Stumpf, & Sar, 1975), and after topical applications of estrogen or vitamin D to skin (Bidmon, Gutkowska, et al., 1991; Bidmon, Pitts, Solomon, Bondi, & Stumpf, 1991; Hayakawa, Kubota, Imai, & Stumpf, 2004).

Examples of autoradiograms are provided in Figs. 1–17 for vitamin D compounds  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$  and its oxygen analog  $^3\text{H}$ -OCT that include target tissues in brain (Fig. 1), spinal cord (Fig. 2), pituitary (Fig. 3), skin (Fig. 4),

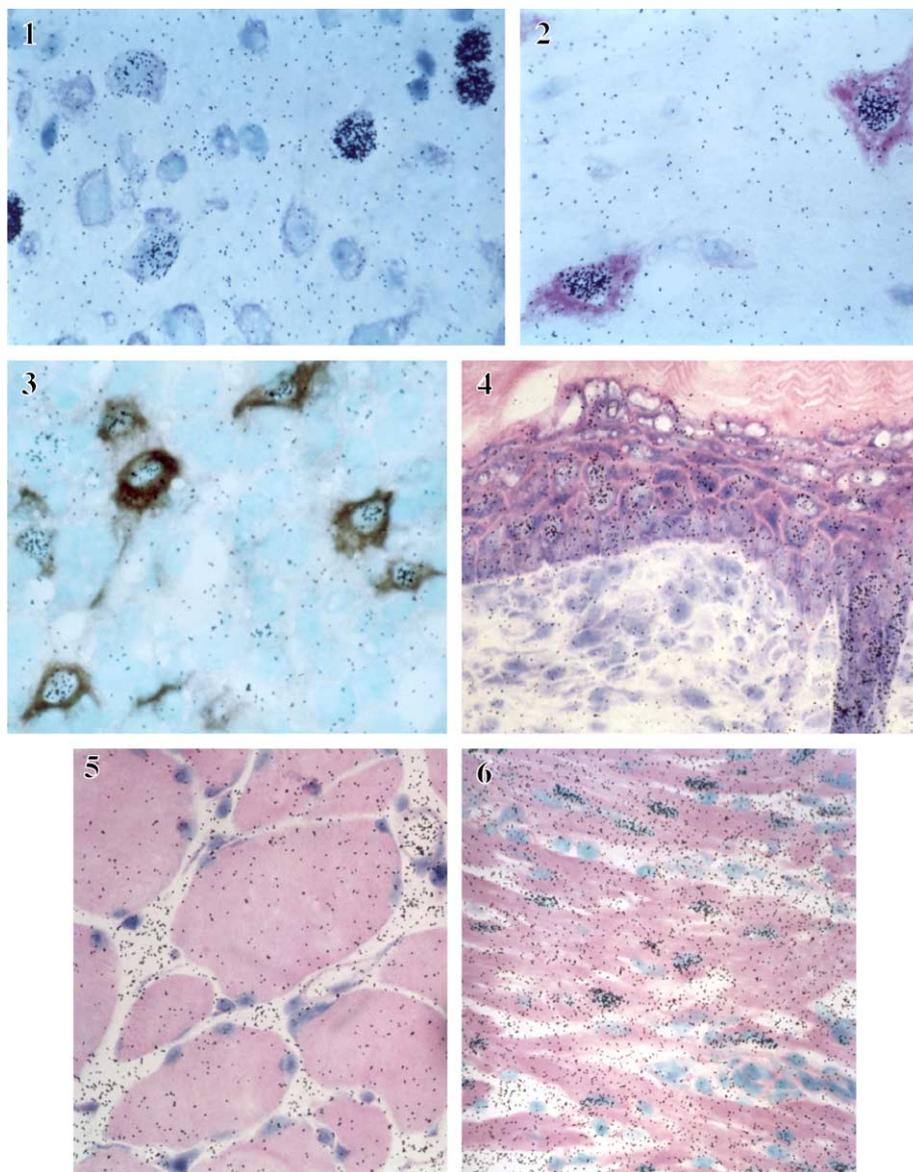


Fig. 1. Example of autoradiogram obtained after injection of  $^3\text{H}$ -labeled 1,25-dihydroxyvitamin D $_3$ , showing nuclear uptake and retention in target cells of central nucleus of amygdala of brain. For details see: Stumpf (1995).

Fig. 2. Example of autoradiogram obtained after injection of  $^3\text{H}$ -labeled 1,25-dihydroxyvitamin D $_3$ , showing nuclear concentration in motor neurons of lamina IX of spinal cord. For details see: Stumpf (1995).

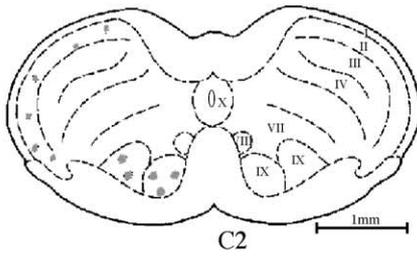
Fig. 3. Example of autoradiogram obtained after injection of  $^3\text{H}$ -labeled 1,25-dihydroxyvitamin D $_3$ , showing nuclear concentration in thyrotropes of anterior pituitary colocalized with antibodies to TSH. For details see: Stumpf (1995).

Fig. 4. Example of autoradiogram obtained after injection of  $^3\text{H}$ -labeled 1,25-dihydroxyvitamin D $_3$ , showing nuclear concentration in keratinocytes of stratum spinosum and basale and hair sheath of skin. For details see: Stumpf et al. (1995).

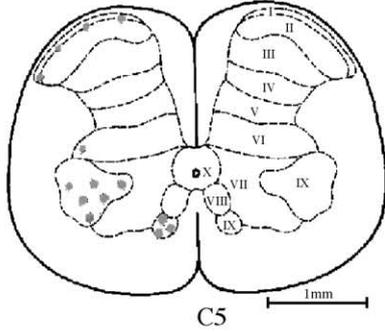
Fig. 5. Example of autoradiogram obtained after injection of  $^3\text{H}$ -labeled 1,25-dihydroxyvitamin D $_3$ . Nuclear concentration is absent in skeletal muscle cells. For details see: Stumpf (1995).

Fig. 6. Example of autoradiogram obtained after injection of  $^3\text{H}$ -labeled 1,25-dihydroxyvitamin D $_3$ , nuclear concentration is present in muscle cells of atrium of heart. For details see: Bidmon et al. (1991).

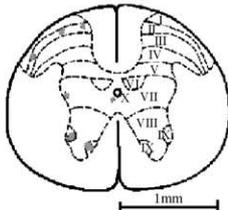
7



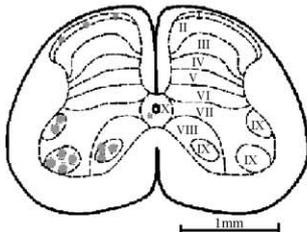
C2



C5

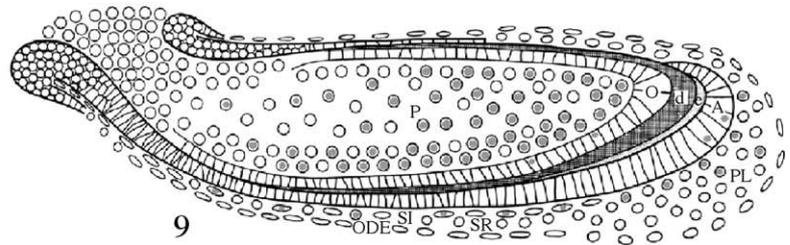
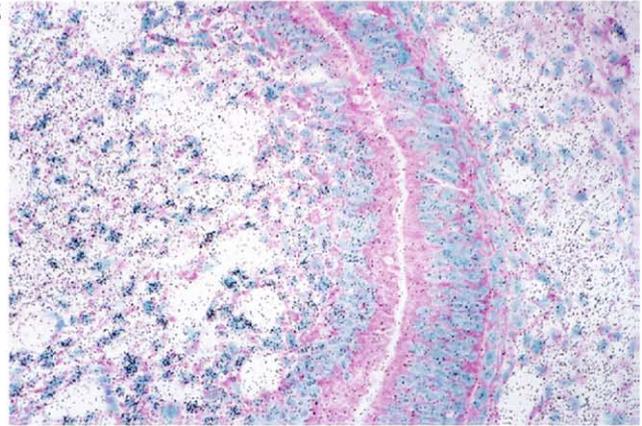


T3



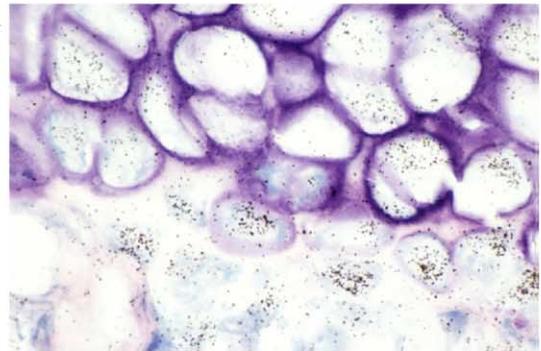
L2

8

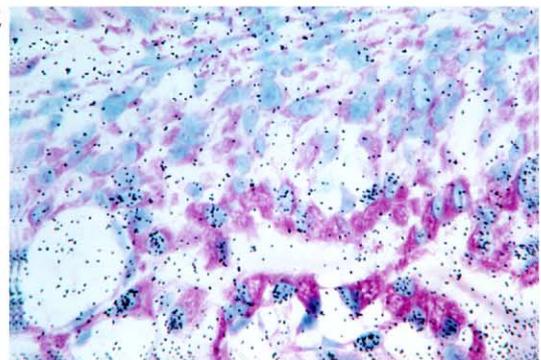


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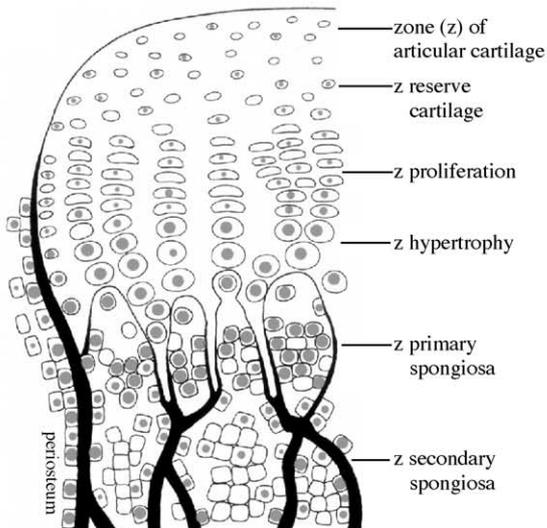
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heart atrium (Fig. 6), tooth (Fig. 8), and bone (Figs. 11 and 12), as well as non-target striated muscle cells (Fig. 5). Examples for the creation of maps after serial section autoradiograms are shown for spinal cord (Fig. 7), incisor tooth (Fig. 9), and tibia epiphysis (Fig. 10). Examples are also given for the quantification of receptor binding with different analogs (Fig. 13), receptor binding saturation with different doses (Fig. 14), and time course of uptake and retention in different tissues (Fig. 15).

By way of comparison, a sample of whole-body autoradiography (Fig. 16) is also provided. It demonstrates the lack of resolution and detail afforded by whole-body autoradiography, which can result in numerous false negatives, either by over- or underrepresenting. For instance, at all time intervals, whole-body autoradiograms show negative uptake of vitamin D compound in brain and spinal cord, which would indicate a blood brain–barrier and negative effects on the central nervous system. By contrast, with receptor microautoradiography, target cell populations and circuits for brain and spinal cord have been discovered (Stumpf, Sar, Clark, Lieth, & DeLuca, 1980), and maps of their distribution published (Stumpf, Clark, O'Brien, & Reid, 1988; Stumpf & O'Brien, 1987), with subsequent evidence for effects on various brain factors such as serotonin (Privette, Stumpf, Mueller, & Hollis, 1991), choline acetyltransferase (Sonnenberg, Luine, Krey, & Christakos, 1986), nerve growth factor (Wion et al., 1991), as well as on mood and related behavior (Landsowne & Provost, 1998).

The discovery of keratinocytes in skin germinal layers, stratum Malpighii and outer hair sheath, as vitamin D targets (Stumpf, Sar, Reid, Tanaka, & DeLuca, 1979), suggested effects on cell proliferation and differentiation. This was confirmed through experiments with skin explants (Hosomi, Hosoi, Abe, Suda, & Kuroki, 1983) and led to the therapeutic use of vitamin D compounds for the treatment of psoriasis (Holick, 1998). These are only a few examples to demonstrate differences between whole-body autoradiography and receptor microautoradiography; many more could be listed.

Other possible applications of receptor microautoradiography include the study of *transporters*. Colocalization of radiolabeled drug and antibodies to transporter may help clarify the role of specific transporters in drug entry to and exit from target cells. Extra- and intracellular time-related distribution may be assessed qualitatively and quantitatively, and the role of various transporters and inhibitors can be evaluated.

For *gene therapeutics*, as with *design drugs* and *tumor drugs* (Koike et al., 1999), information can be garnered on whether and to which degree target cells can be reached, as well as on the distribution of 'target' versus 'nontarget' cells in tumors.

Routes of drug delivery, penetration, and absorption may also be studied with receptor autoradiography, both in vivo and in vitro.

### 3. Discussion

Various topics related to the selection of procedure in drug research and development and related consequences deserve consideration. This includes *expediency* that is a dominant feature in current ADME studies. Expedient procedures that procure data quickly have priority. However, expediency should not be determined primarily by speed, but above all by values, such as authenticity and utility of data. The most speedy procedure may turn out to be less expedient than seemingly nonexpedient but thorough and more informative ones. A careful and balanced selection of approaches is indicated.

#### 3.1. Validation and selection of procedure for drug localization and targeting

Different methods for in vivo drug localization are recommended in the literature and routinely applied without sufficient testing of their utility and limits. Misleading data have confused investigators by diminishing the accuracy of diagnosis and prediction (Monro,

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Fig. 7. Example of map prepared from autoradiograms after injection of  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$ , showing different levels of rat spinal cord (after Stumpf et al., 1988). For details see: Stumpf et al. (1994).

Fig. 8. Example of autoradiogram after injection of  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$ , showing a section through incisor tooth with nuclear uptake and retention, predominantly in pulp cells. For details see: Kim et al. (1985).

Fig. 9. Example of map prepared from autoradiograms after injection of  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$ , showing a section through incisor tooth with nuclear uptake and retention, predominantly in pulp cells. For details see: Kim et al. (1985).

Fig. 10. Example of map prepared from autoradiograms after injection of  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$ , showing bone of epiphyseal region of rat tibia. For details see: Stumpf et al. (1994).

Fig. 11. Example of autoradiogram after injection of  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$ , showing bone of epiphyseal region of rat tibia with nuclear labeling of hypertrophic chondrocytes. For details see: Stumpf et al. (1994).

Fig. 12. Example of autoradiogram after injection of  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$ , showing nuclear concentration in osteoblasts surrounding a bone spicule. For details see: Stumpf et al. (1994).

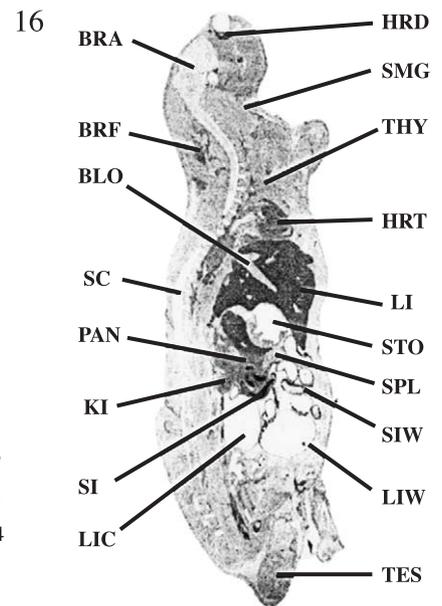
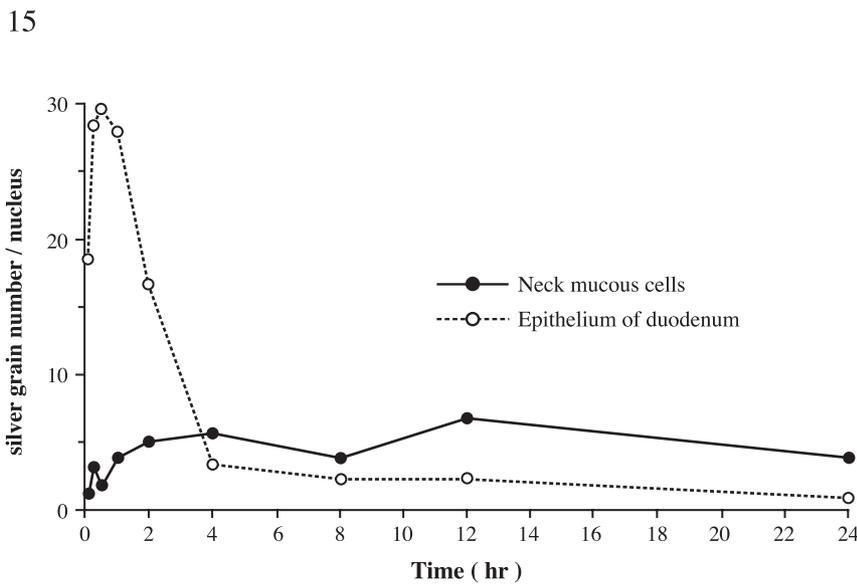
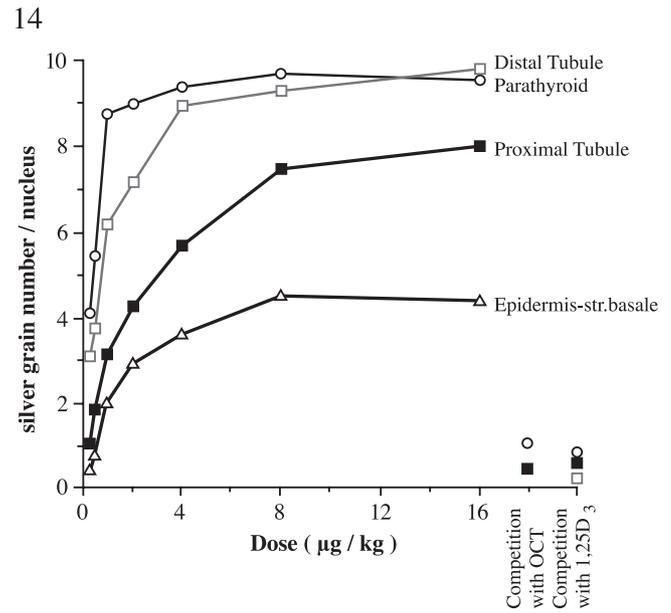
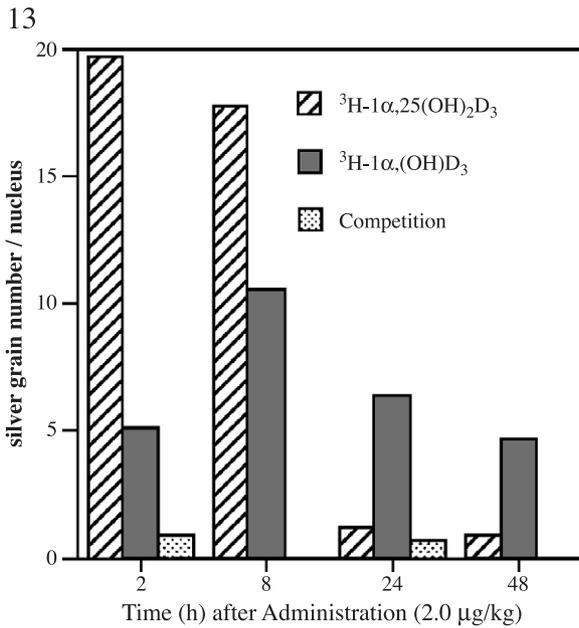


Fig. 13. Example of quantitative evaluation of autoradiograms after administration of  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$  or OCT analog. This figure shows the difference in cellular uptake and release of radioactivity in tibia osteoblasts, which is rapid after  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$ , but delayed and sustained after  $^3\text{H}$ -1 $\alpha$ (OH)vitamin D $_3$  (Koike, Ishikawa et al., 1998).

Fig. 14. Example of quantitative evaluation of autoradiograms after administration of  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$  or OCT analog. This figure depicts differences of nuclear uptake and saturation among different target tissues for vitamin D (Koike, Hayakawa et al., 1998).

Fig. 15. Example of quantitative evaluation of autoradiograms after administration of  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$  or OCT analog. This figure compares nuclear uptake and retention of radioactivity between epithelium of duodenum and neck mucous cells of the isthmus region of gastric glands, indicating considerable differences between these target tissues after injection of  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$  or its OCT analog (Stumpf, Koike, Hayakawa, Hirate et al., 1995). Isthmus cells in the stomach give rise to parietal cells, chief cells, and surface epithelium, suggesting a role of vitamin D in cell renewal and differentiation.

Fig. 16. This figure provides one level of whole-body autoradiogram prepared under conditions comparable to those for receptor microautoradiography shown in Figs. 1–6, 8, 11, and 12. Notice the differences in resolution, when compared to the data obtained with receptor microautoradiography. Limitations of resolution and false negatives with whole-body autoradiography are apparent (Stumpf, 2003).

1994). Years of hard work using misleading methods may result in incomplete or even false data. Years of further hard work by careful investigators may then be required to disprove false claims made in the literature (Chamness, Mercer, & McGuire, 1980).

Repeatedly, we have postulated that any “new” technique for drug localization, *before* being introduced, should be validated as to its authenticity of localization and resolution with at least two diffusible compounds for which tissue and cellular localization have been established through alternate means. We have followed this dictum before recommending and applying our dry-mount and thaw-mount autoradiography (Stumpf & Roth, 1966). Such basic tests have not been performed with whole-body autoradiography, *in vitro* section incubation autoradiography, and various other *in vivo* scanning procedures. Therefore, claims cannot be made that these methods are useful for demonstrating the tissue localization of drugs (Stumpf, 2002, 2003). Such all-encompassing and generalizing use of “the” is unsupported—it can at most mean “some” but not “all”. Related assumptions can be misleading if limitations in sensitivity and resolution, along with resulting false negatives, are not taken into consideration.

Drug companies have refrained from the regular use of microautoradiography, perhaps, for several reasons: inadequate methods may have been applied with related inability to distinguish results from artifacts; inertia in using old methods and unwillingness to spend time and money to gain expertise in a new method that requires diligent application; lack of pressure from regulatory agencies; and a lack of appreciation of the importance of structural–functional detail.

Irresponsible advertising, statements by incompetent and inexperienced investigators, and uncritical use of untested procedures continue to be harmful. As an example, a CRO recently advertised drug testing with microautoradiography based on the use of cryosections up to 10  $\mu\text{m}$ , including liquid fixation and liquid emulsion coating, all of which are potential sources of loss of compound, diffusion, redistribution, and other kinds of artifacts. Using a wrong technique, much time and energy will be wasted, in addition to the more serious profusion of false and misleading data.

Considering the criteria mentioned, the experience of this author, and the published evidence, receptor microautoradiography is the only tested and reliable method currently available for the microscopic cellular and subcellular *in vivo* localization of drugs. Various modifications of autoradiographic procedures for diffusible compounds have been recommended in the literature (Mizuhira, Shiihashi, & Futaesaku, 1981; Nagata, 2002; Appleton (Baker, 1989); Kawamoto, 1990, 2003). While all appear to offer improvements in single procedural steps, however, these and other methods have not stood the test of time. Some of the recommended methods seemed to be useful in certain applications, but not in others. Artifacts, sometimes unrec-

ognized as such, were published as results (Stumpf, 1969; Stumpf & Pilgrim, 1995).

While the modifications recently proposed by Kawamoto appear to improve resolution over the 20- to 50- $\mu\text{m}$ -thick sections that are commonly used in whole-body autoradiography, deleterious steps during freezing, mounting, and handling of tissues, the application of glue and tape limits its general applicability beyond the study of hard tissues. Slow freezing of a whole animal, as applied for whole-body autoradiography, is associated with intra- and extracellular ice crystal formation and related disruption of tissue structure to a degree that precludes useful microscopic cellular and subcellular resolution, even if the section thickness could be reduced to 1  $\mu\text{m}$ . It remains to be demonstrated, as claimed by Kawamoto, that the same tissue used for whole-body autoradiography can generally also be used for microautoradiography.

*In autoradiography, the picture is the evidence.* A review of published autoradiograms, not mere quantification, not only singular application but repeated applications of the same technique, and a comparison of results among different techniques applied to the localization of drugs, would reveal whether a designated technique is indeed useful and the claims made for it are justified.

*Immunocytochemistry* with antibodies to drug has been attempted as an alternate high resolution approach (Jungblut & Sierralta, 1998) to localize drugs, but with limited success (Stumpf, 1999). While it is possible to obtain specific antibodies to drugs, obstacles remain, both in retaining and immobilizing drugs at their *in vivo* sites of deposition and in excluding artifacts of translocation and loss during treatment with fixatives, antibodies, and rinsing. Receptors, unlike drugs, are large proteins and can be retained using liquid fixatives and then localized with antibodies. However, can receptor localization alone be used as a guide for drug effects? While sites of receptors and sites of drug binding may correspond in some areas, they may not be identical in others. Differences may exist because of variations in receptor–drug affinities, uptake, retention, and other factors. Moreover, presence of receptor protein does not necessarily imply binding (Gasc et al., 1983). Moreover, sensitivities of the two methods of microscopic localization using radiolabeled drug and using antibodies to related receptor, are not fully identical (Zorn, Soto-Suazo, Pellegrini, Oliveira, & Stumpf, 2003). Available evidence renders it unlikely that antibodies to receptor can replace radiolabeled compounds for the identification of *in vivo* drug targets.

### 3.2. Prediction of drug action

*Predictions* of drug actions become more reliable when data on specific *in vivo* tissue and cellular localization and related kinetics are available and taken into consideration (Boulnois, 2000). Interactions with receptor proteins underlie most drug effects. In general, the drug needs to reach the receptor site for an action to initiate, as is expressed in the rule

“corpora non agunt nisi in loco” (substances do no act, unless on site—Stumpf, 1996). Effects unrelated to cellular receptor binding may exist. In either case, information about sites of drug deposition and receptor binding are not only of interest but also needed to aid understanding and prediction.

Nonetheless, methods routinely employed for prediction lack sensitivity and resolution, albeit expedient and satisfactory for regulatory requirements, and miss important information. Symptomatic is the fact that kinetics of radioactivity levels in blood and other body fluids, as well as in whole organs or chunks of organs, are currently used as a basis for prediction and related modeling, despite evidence that kinetics of drug binding to target tissues may be quite different from kinetics of blood and even vary among different target tissues (Koike et al., 1998).

### 3.3. Bioavailability

*Bioavailability* of a drug is commonly assessed through time-related measurements of blood levels after administration. The term itself and current semantics (see <PharmPK@boomer.org>) assume that the blood level of a drug, modified by unbound versus bound fraction, is available for action. However, in light of the published documentation and related considerations in this review, it follows that “bioavailability” of a drug assessed from systemic blood measurements should not be used as an indicator for target availability. No evidence has been provided in the literature to show a general correlation between drug levels in blood and specific target cell populations. Data from mixed population organs with large unspecific tissue components, while frequently utilized, are not suitable. In vivo measurements of drug-receptor pharmacokinetics in addition to that of blood are required to establish valid whole-body pharmacokinetic models. “Modeling results should always be interpreted taking into account where the intended site of action (useful or adverse) is” (Nestorov, 2003).

Bioavailability needs to be determined not only in blood or whole organs or chunks of organs but also separately and selectively in target tissues, i.e., individual target cell populations. For instance, measurements of “kidney” would miss important differences among its functionally diverse parts, such as podocytes, distal and proximal tubules, and others. Functional differences are most conspicuous in neuronal circuits and diverse cell populations of brain and spinal cord, but exist also elsewhere, even in the “heart” (Stumpf, 1995; Stumpf, Sar, & Aumueller, 1977). The complex composition and diversity of cell populations in any organ renders it fallacious to use lumped organ data for target identification as a basis for modeling and prediction.

### 3.4. Drug homunculus

A drug-target homunculus, as shown in Fig. 17 for vitamin D, represents an overview of in vivo drug-receptor

(target) binding projected onto a schematic human body. The concept of “drug homunculus” evolved from the detailed information provided through receptor microautoradiography with estrogens, progestins, adrenal corticoids, retinoic acid, thyroid hormone, and, especially, vitamin D compounds (Stumpf, 2003). A drug homunculus can simultaneously represent detailed target locations and overviews for comparisons of drug-specific deposition, binding sites, and related functions. Such a visual composite facilitates rapid recognition of hierarchical sites of action, potential side effects, and toxicity. Accompanying computer links can further inform about target-associated biological effects that may include results from in vitro tests, clinical and behavioral parameters, and any other relevant information.

A drug homunculus can be used for characterizing and fingerprinting any drug, design drug, analog, or antagonist, as well as serve as a resource for drug research and development, for documentation and submission to regulatory agencies, and for information to medical doctors and patients.

### 3.5. Low-dose versus high-dose effects: hormesis and toxicity

The *Schultz–Arndt rule*, which was conceived more than a hundred years ago, indicates that “toxic” compounds, known to be inhibitory and lethal at high dose, are stimulatory and beneficial when administered at low dose. This paradigm applies to many compounds. There is a need to investigate low-dose effects of “toxic” compounds for exploratory and practical reasons. Paracelsus, when accused of using opium, then perceived as a poison, for treatment of patients, made the fundamental statement that “there is nothing that is not a poison” as “the dose makes the drug”.

Pharmacologists have neglected the study of hormesis (Calabrese & Baldwin, 1999; Doull, 2001), the term used to describe low-dose stimulatory effects of toxic agents. One of the reasons for this omission and the focus on high-dose effects lie in the difficulty recognizing and investigating low-dose effects. Studies of low-dose effects demand attention to detail, patience, and high-resolution–high-sensitivity approaches. As such, valuable information on the phenomenon of hormesis may be gained with high-resolution receptor microautoradiography. With radiolabeled compounds of high specific activity, target sites of low-dose deposition and action, which may well differ from those of intermediate and high doses, can be identified and characterized. As argued above and demonstrated through various studies, low-dose sites remain hidden, all or in large part, when sought by current conventional procedures, such as radioassay, whole-body autoradiography, nuclear imaging, and biochemical analysis of homogenized tissue.

An interesting observation from our autoradiographic studies that beckons follow-up is: with various steroid hormones, cellular and subcellular localization of radio-

## RECEPTOR DRUG HOMUNCULUS

## Vitamin D Target Tissues

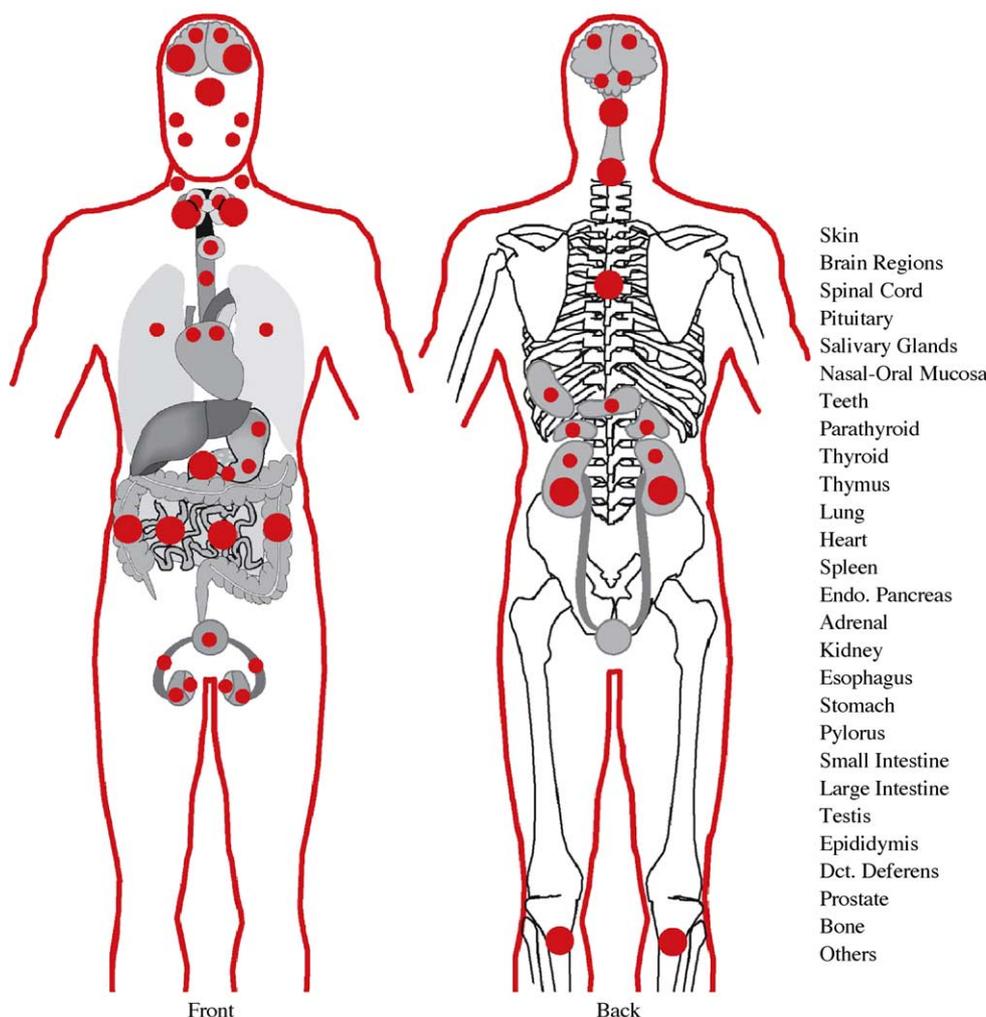


Fig. 17. Vitamin D target tissue distribution—Drug Homunculus—based on results from receptor microautoradiography with  $^3\text{H}$ -1,25(OH) $_2$ vitamin D $_3$  and its analog  $^3\text{H}$ -OCT, studied in male rats, mice and hamsters and transposed to a human organ schematic for surveys and clues for sites of action, ‘side’ effects and toxicity. Target tissues with nuclear concentration are marked as red dots (after Stumpf, 1995, 2003). Many of these target tissues have been confirmed and characterized through biochemical, histochemical, and clinical follow-up.

labeled compound varied in relation to dose and time. Cellular concentration and retention in target cell populations was not only nuclear. With near-physiological doses, nuclear receptor binding could be well recognized and genomic target tissues could be well monitored. By contrast, with elevated doses, nuclear uptake became saturated and extranuclear deposition increased and finally prevailed, so that nuclear concentration was then no longer apparent; but rather, cytoplasmic and extracellular radiolabeled compound dominated. These observations indicate that target nuclei stand out as sites of high avidity with low and intermediate doses, while the same target cells may appear as sites of equal or negative uptake with high doses. Such changes of hormone and drug deposition probably are related to initial binding and saturation of primary sites first, followed by predominant occupation of secondary low-specificity-high-

capacity sites as the dose increases. Secondary sites with “high capacity” may include transport proteins, as well as cellular components that account for certain pharmacological and toxicological effects.

It is even conceivable that an increasing dose and related change of deposition and binding can reach a *threshold*, after which a *reversal of effects* is correlated. For instance, in the case of vitamin D and skin, the stimulatory effect on cell proliferation and differentiation with near-physiological doses (Hosomi et al., 1983; Tian, Chen, & Holick, 1995), correlating with nuclear concentration in keratinocytes (Stumpf et al., 1979), may be reversed to reach the inhibition of cell proliferation, as is apparent with high-dose treatment of psoriasis (Holick, 1998). The prevailing cytoplasmic deposition and retention arising from a high dose—observed in autoradiograms after topical application

(Hayakawa et al., 2004)—correlates with inhibitory rather than stimulatory effects on the genome, possibly involving stimulatory intermediate actions on cytoplasmic and/or plasma membrane associated components. A biochemical and molecular follow-up of such in vivo observations is suggested to include various target tissues. The same rationale of hormetic reversal of action may apply to the treatment of vitamin D target tumors. A differential cellular uptake and retention of the oxygen analog OCT has been demonstrated in xenografts of human pancreatic tumor cells with receptor microautoradiography (Koike et al., 1999).

### 3.6. Conclusion

As made evident by this review, in vivo localization of drugs with receptor microautoradiography offers critical details about tissue targets that are relevant to molecular mechanisms of action and toxicity, but are difficult or impossible to achieve otherwise. Receptor microautoradiography provides qualitative and quantitative data about cellular and subcellular components at high magnification as well as integrative overviews of whole organs or regions of organs at low magnification, both with the same preparation. It is a sensitive tool for *target identification* and *target validation* in drug research and development. Through receptor microautoradiography, unique information and essential leads for *diagnosis* and *prediction* can be obtained.

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