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# PLANE-DEPENDENT (RELATIONAL) MORPHOMETRY IN HUMAN LUNG CARCINOMA: A SUITABLE TECHNIQUE FOR DELINEATION OF STRUCTURE-ASSOCIATED STEREOLOGICAL FEATURES

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## ABSTRACT

Vascularization- and cell proliferation-associated features convey potentially valuable characteristics about human malignancies for the pathologists. Pathologists are obliged to develop suitable techniques and approaches to exploit the preserved information to its complete extent. Using stereological tools, commonly only one reference unit is chosen for the estimation of particle (volume) densities Vv, surface densities Av, or numerical densities Nv (numbers of structures by volume). However, an image can be divided into multiple different compartments which might be used to serve as independent reference spaces. The technique described in this article is based upon the segmentation of certain "basic structures" and the use of their center of gravity as reference center for the establishment of a structure-associated reference plane (space). The surface of the segmented structure serves as inner reference boundary. The boundary will then be artificially expanded with a defined perpendicular diameter as defined by the shortest inner perpendicular diameter (center of gravity to surface), and a (new) outer reference boundary is created. This computed space serves for reference space for particles, structures etc. located within the new reference plane. By expansion of the inner and outer reference boundary volume several stereological features can be assessed with respect to "distance to surfaces", features of or relations between the basic structures. The general principle and the derived mathematical equation will be explained. In addition, the results of first measurements performed on vascularization in human lung carcinomas are presented.

**Key words:** relational reference space, vascularization, proliferation, lung carcinoma.

## INTRODUCTION

The classical stereological tools include in general the use of a reference plane A (volume V) (for example size of an examination plane of a histological slide), the selection of an appropriate grid with well defined distances of "test lines" ("points"), and the counting of the number of intercepts "hits" x of the test lines (points) with the areas of the chosen objects or

their outline [Gunderson et al, 1988a,1988b; Underwood, 1970]. From the relation x/A (x/V) the basic parameters such as area density (Aa), volume density (Vv), boundary density (Ba), numerical density (Na), surface density (Sv) can be estimated [Underwood, 1970]. These procedures are well established, and have been proven readily exploitable in anatomy, pathology, mineralogy, and biology [Gunderson 1988a, 1988b; Collan and Mariuzzi, 1995; Costello et al, 1995; Karlson and Gokhale, 1996; Sikora et al, 1996; Underwood, 1970]. Only one reference plane (space) is required for the calculation of parameters with the exception that the total reference space can be divided into a space of "interest" or reference compartment and an additional space not included in the reference space (external space). A graphic example for referring organ compartments to these categories is the interstitial lung tissue (reference space) and the associated air spaces (alveoli). The assessment of the mentioned parameters is then performed by calculating the relative numbers of hits within the reference space and the external space as well as the volume ratio of the two spaces. Since the given approach is not limited to histological problems, it is instructive to describe its space by posing a problem which has no connection to pathological practice:
If e.g. we want to answer structure-associated questions such as "what is the car density in relation to the distance from the cities ?" or "what is the average number of apartments in a house in relation to the distance from the city center ?", it seems appropriate to "construct" several different virtual reference spaces with a "projection center" (center of the various cities) and to estimate the numerical densities of cars (apartments/houses) within each of these reference spaces. We then have the stereological parameters which we can compare and use for further computations and derivations. In the following, vessels present in human lung tumors have been chosen as "projection centers", and examples of questions (and corresponding measurements for providing adequate answers) are presented.

## BASIC IDEA AND FORMULAS

A metric two dimensional plane A, and certain objects Oi located within A are given. The existence of objects implies that a procedure S exists with

$$S(O_i) = \{c_i, B_i\}, \text{ and } \{O_i\} \cap \{A - \{O_i i\}\} = 0.$$

$S$ is called segmentation, and $c_i$ represent the geometrical (gravity) centers of the objects with the object space $O_i$ and its boundary $B_i$. We can then choose $\{A-\{O_i\}\} = Ro$ for a new reference space $Ro$. If we have, in addition, "particles" $P_j$ (for example cells, nuclei, etc) located in $Ro$ we can estimate the stereological parameters $SPs(P_j,Ro,Fs)$ in $Ro$, in case we select $Ro$ as reference space and apply the adequate stereological equations Fs. For example, the area density would be

$$SPs(P_j,Ro,Fs) = Aa(x,Ro); \qquad (1)$$

where $x$ = number of intercepts ("hits") of the particles $P_j$ with a chosen test system $a$ in the reference compartment $Ro$.

In a next step, we can expand this procedure, and construct an "artificial" object ***Oai*** with a center $ca_i$, and a boundary $Ba_i$. If we demand $O_i \subset Oa_i$, having the same center of gravity ci, and $B_i \subset Ba_i$, we have a new object $Oa_i$ with a specific and fixed geometrical relation to the embedded object $O_i$. We can now choose the object $Oa_i$ as a new reference space ***R1***, and calculate the stereological parameters ***SPs($P_j$,R1,Fs)*** of the particles $P_j$. Equation (1) will then become

$$SPs(P_j,R1,Fs) = Aa(x,R1); \quad (2)$$

where $x$ = number of intercepts ("hits") of the particles $P_j$ with a chosen test system a in the reference compartment ***R1***.

We can then perform the same procedure with a second reference space ***R2***:

$R1 \subset R2$, $c1_i = c2_i$, and $Ba1 \subset Ba2$, and fully repeat this procedure with other selected reference spaces of choice.

When applying the same stereological procedure ***Fs*** on the "particles" $P_j$, the set of parameters ***{SPs}*** depends only upon the different reference spaces ***{R1,R2,...}***, or, in other words, upon the geometrical relationship of the chosen virtual reference spaces. This procedure is a convenient and versatile technique to analyze the geometrical relationship of stereological parameters in a given two dimensional scene. Its application will be illustrated for a certain property of malignancies.

## MATERIAL AND METHODS

The described approach has been applied to histological slides of resected lung carcinomas. The slides were subjected to monitoring of vessel presence by a monoclonal antibody against factor VIII [Sinowatz and Plendl, 1996] and of spatially associated tumor cell nuclei. The deparaffinized and rehydrated slides were incubated with Texas-red-labeled avidin for detection of binding sites at room temperature for 30 min. Afterwards, the nuclei were stained with DAPI, and the tissue sections were analyzed using a fluorescence microscope equipped with a highly sensitive one chip black and white TV camera (Leica, Bensheim, Germany) connected to a frame grabber (Leutron XFP, Munich, Germany). Two corresponding images were collected by moderate magnification (x25 objective) with an absorption filter system for Texas-red (Filter block TX13833, vessels, applied probes) and for DAPI (Filter block A513824, DNA content [Wand et al, 1995; Kayser et al, 1994a; 1996] and position of cells to be analyzed). An interactive image analyzing system (DIAS, Powersoft, Berlin) for the stained nuclei was used. Knowing the position of the cell nuclei, the staining intensities of the nuclei were then automatically calculated. The vessel walls in the histological slides were marked interactively, and the vascular boundary was approximated by an ellipse. The partial virtual reference volumes were constructed by expanding the boundaries for 40, 80, >80 μm calculated for the minimum diameter of the ellipse. The whole procedure is depicted in Fig.1. All in all 60 surgical specimens of primary lung carcinomas have been processed. Only lobes and lungs with potential curative resection were included in this study. A detailed macroscopic examination including the analysis of at least one complete tumor cross-section, accurate post-surgical TNM classification, and a determination of the tumor cell type according to the rules of the WHO was routinely performed [WHO, 1982]. The material comprised 30 epidermoid carcinomas, and 30 adenocarcinomas. The tumor cell classification

was based upon HE, PAS, Feulgen-stained slides, and on immunohistochemical techniques, if necessary. A random sectioning (nucleator) of the tumors according to the technique described by [Gunderson et al, 1988a; 1988b] was performed.

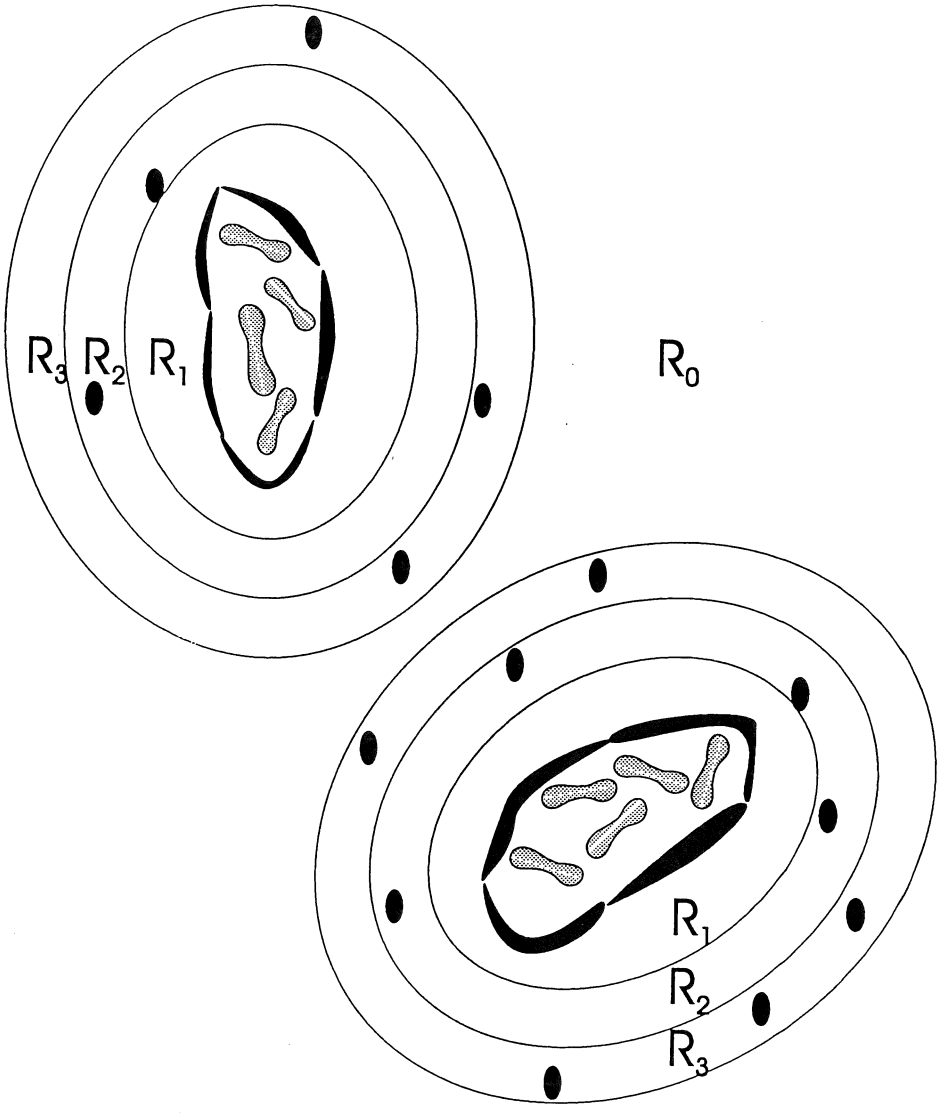


*Fig. 1. Scheme of two segmented vessels demonstrating the construction of virtual surface-related reference spaces R1, R2, R3. Only cells whose nuclear centers of gravity are located within the corresponding reference space are taken for further calculations of stereological parameters such as numerical densities, volume fractions, etc.*

## PROGRAM STRUCTURE AND PERFORMANCE OF MEASUREMENTS

The program based upon the DIAS software (Towersoft, Berlin) comprises several modules. These include:

1. Image acquisition and storage: Two images are grabbed in accordance with the different fluorescent length wave (Texas red for marking of the vascular walls or endothelial cells, and DAPI for quantitative measurement of the integrated nuclear fluorescence (INF).

2. Image segmentation: The images are inverted and boundaries of the stained endothelial cells and nuclei of tumor cells are segmented. The circumference of a vessel is then approximated by an ellipse. After construction of the ellipse and computation of its area, its circumference is virtually expanded in 40 microns steps by increasing the minimum diameter. The maximum diameter is expanded in relation to the ratio (maximum/minimum diameter), and the boundary of the new ellipse is computed. All pixels located within the area of expansion will be marked by a specific "overlay color" which serves for the calculation of the virtual ellipse size. The procedure is performed simultaneously for all vessels. Pixels which have already been marked by an overlay color are excluded from further marking; i.e., only the nearest distance of a pixel to a vessel is taken into account.

3. Measurements of the Integrated Nuclear Fluorescence (INF): The tumor nuclei are marked interactively, and the nuclear boundary, the nuclear size, and the INF are calculated. Furthermore, the center of gravity of each nucleus is computed. The nucleus is included into the vascular compartment in accordance with the "overlay" marking of its center. The INF of lymphocytes is then measured in an identical procedure.

4. Classification of INF distributions: The next step is the calculation of frequency distribution of the INF of measured tumor cells and lymphocytes. The 2C peak of lymphocytes serves for reference, and the INF of tumor cells is classified as follows: S-phase related INF fraction (SINF): 2.75<INF<3.25; percentage of tumor cells with an INF>5C (INF5C): INF>5.0.

5. Stereological computations: The modules described above provide all data which are needed for further calculations: The volume fractions and the surface fractions of the vessels were computed as usual. The increase in size of each expanded vessel (virtual ellipse) serves for the new reference area (volume) and the number of included tumor cell and lymphocyte nuclei are computed by counting the marked pixels and centers of the nuclei dependent upon the overlay markings. Only nuclei whose centers are located within the corresponding virtual reference area are taken into account. The numerical densities of the different INF classes of tumor nuclei and lymphocytes are computed as usual, and presented in a data file for further statistical analysis.

## RESULTS

Synopsis of the material is given in Table 1. The surgical specimens of 60 patients have been included in this first study. The mean age of the included patients is comparable to that of all patients operated on for lung cancer.

The dependence of numerical density of tumor cells with 2.75 < INF < 3.25 (S-phase-related tumor cell fraction), and that of cells with an INF>5C (aneuploid cells) upon the distance from the vascular walls is listed in table 2. Cells of the S-phase-related tumor cell fraction are frequent near the vascular boundary, those with an INF>5C in the distant compartments.

*Table 1: Synopsis of material.*

| | N (number of tumor samples) | Mean age and standard deviation |
|---|---|---|
| **Sex** | | |
| - male | 69 | 61 ± 9.8 |
| - female | 21 | 52 ± 10.2 |
| **Cell type** | | |
| - epidermoid carcinoma | 30 | 64 ± 10.5 |
| - adeno carcinoma | 30 | 58 ± 10.2 |

## DISCUSSION

Stereology is an established and well-appreciated technique for the determination of cellular and textural parameters, especially volumes and volume-associated numerical densities [Collan and Mariuzzi, 1995; Gunderson et al, 1988a; 1988b; Underwood, 1970]. Application of syntactic structure analysis can provide additional insights into two-dimensional features of biological material such as human lung carcinoma or development of fetal organs [Kayser et al., 1996; Kayser and Gabius, 1998]. The outlined approach combines structural features with those obtained by stereological techniques, and can be instrumental to quantitate immuno/ligandohistochemical staining intensities. The basic idea is to divide the reference space into several, not necessarily complementary "sub-reference spaces" which then are subjected to the assessment of stereological parameters. If these sub-spaces represent biologically meaningful structures and if they are chosen within their natural tessellation, the obtained sub-volume fractions can be sub-summarized according to the textural relationship of the reference spaces. The first demonstration addresses a simple question: is the proliferation and heterogeneity of a certain human lung carcinoma associated with the "assess to a source of nutrition" for the tumor cells, i.e., the distance of the tumor cells from the vascular surface of the nearest vessel (capillary, arteriole) ? This question is related to the reported association of tumor neovascularization and the prognosis of patients [Costello et al, 1995; Fontanini et al, 1995; Sikora et al, 1996; Visscher et al, 1993], and can be answered as follows: The proliferation as measured by the volume fraction of the S-phase-related tumor cell fraction is increased in those tumor cells located close to the boundary of the vessels, especially within a distance < 40 μm. The opposite holds true for tumor cells loalized far away from the vascular surface (> 80 μm). Within these reference spaces the volume fraction of tumor cells with an INF > 5C is increased, and that of the S-phase-related tumor cell fraction decreased. The data are readily interpreted: the better the access to a source for nutrition, the more likely any biological system is placed in the reduplication stage.

The second approach employs the analysis of textural features for the construction of the reference space in order to characterize sub-units within this area, for example volume fractions of binding sites to certain ligands. As only positive signals can be measured, and a negative signal is equal to zero, an appropriate monitoring of the reference space (cellular area) is a prerequisite for any meaningful measurement. The neighborhood condition as given by the minimum spanning tree is a pertinent and reproducible technique, if a maximum volume, in addition, is introduced [Kayser et al, 1995b]. In cytological smears, the technique of the determination of the Voronoi pattern as proposed by [Guiilaud et al, 1997] is not practicable because we are generally dealing with singular and isolated cells. It should not be overlooked

that the actual measurements of this investigation primarily served the purpose to demonstrate the practicability of the approach rather than to evaluate definite results.

In summary, the construction of relational reference spaces offers a potent parameter for calculation of texture-associated stereological features.

*Table 2: Mean values of vascular features (minimum diameter, volume fraction* Vv, *surface fraction* Sv*), and of nuclear features (numerical densities* Nv*) in the various virtual reference compartments (distances to vascular surface).*

| ***Feature*** | ***Mean*** | ***Standard deviation*** |
|---|---|---|
| Volume fraction of vessels (Vv); ($\mu m^3/\mu m^3$) | 12.5 | 7.8 |
| Surface fraction of vessels (Sv); ($\mu m^2/\mu m^3$) | 1.7 | 0.6 |
| Minimum diameter of vessels ($\mu m$) | 14 | 8 |
| **Numerical density of S-phase-related fraction Nv(SINF)/numerical density of tumor cells Nv(Tumor)** | 0.1 | 0.03 |
| - Nv(SINF)/Nv(Tumor) within the virtual reference space I (<40 $\mu m$) | 0.1 * | 0.2 |
| - Nv(SINF)/Nv(Tumor) within the virtual reference space II (41 - 80 $\mu m$) | 0.07 | 0.02 |
| - Nv(SINF)/Nv(Tumor) within the virtual reference space III (>80 $\mu m$) | 0.06 | 0.02 |
| **Numerical density of tumor cell fraction with an INF>5C Nv(>5C)/numerical density of tumor cells Nv(Tumor)** | 0.4 | 0.1 |
| - Nv(>5C)/Nv(Tumor) within the virtual reference space I (<40 $\mu m$) | 0.3* | 0.1 |
| - Nv(>5C)/Nv(Tumor) within the virtual reference space II (41 - 80 $\mu m$) | 0.4 | 0.1 |
| - Nv(>5C)/Nv(Tumor) within the virtual reference space III (<80 $\mu m$) | 0.5 | 0.2 |

Explanations: * : difference statistically significant ($p<0.05$)

Virtual reference spaces : the numerical densities Nv are calculated for those cells only whose nuclear centers of gravity are located within the corresponding distance from the nearest vascular surface (i.e., I : < 40 $\mu m$; II: 41 – 80 $\mu m$; III : > 80 $\mu m$).

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## APPENDIX

The above discussed general procedure, which divides a given image into several non-overlapping virtual reference areas can, in addition, be applied to various problems of related nature. One of these problems is the quantification of cytoplasmic immuno/ligandostains. In this approach, the image is segmented into several virtual reference spaces which rely to the estimated size of cells. The algorithm can be subdivided into two subsequent procedures, namely a) the calculation of a virtual cytoplasm size, and b) the computation of the staining intensity within this (virtual reference) area.

## CALCULATIONS OF VIRTUAL CYTOPLASMIC SIZE

In principle, the quantitation of staining intensities in immuno/ligandohistochemically stained slides is difficult as the size of the object to be measured is not known (for example of negative cells) in general. Therefore, the size of the objects to be measured has to be inferred by employing appropriate algorithms. In our approach, the following constraints have to be fulfilled, if the volume fraction of "positive" (stained) cells $Vvp$ and their intensities are to be calculated:

1. We have to be able to localize (define) each object (cell) within the area of interest (for example by segmentation of the nuclei).
2. We have to know the average size of the objects (area of cells).
3. We have to define the nearest neighboring cells by an appropriate algorithm.

If these prerequisites are fulfilled, we can apply the following equation for the calculation for the stained cells:

$$Ic_k = 1/Nv \quad \text{if} \quad \Sigma[\Delta(Ic_i, Icb)] - Rc*(DIcm) > 0 \qquad (3)$$

$$Ic_k = 0 \quad \text{elsewhere.}$$

We then can calculate

$$Nvp = \Sigma (Ic_k)$$
$$Vvp = Vmp * Nvp * Nv$$

***Vmp*** = mean corpuscular volume fraction of "particles", i.e. cells.
$Ic_k$ = staining intensity of cell ***k***.
$Ic_i$ = staining intensities of compartment (pixel ***i***) within the particle (cell) ***k***.
***DIcm*** = background of staining intensity within the restricted reference space (cell).
***Icb*** = background intensity (outside the restricted reference space (cell).
***Nv*** = numerical density of all cells.
***Nvp*** = numerical density of stained cells.
***Vvp*** = corpuscular volume fraction of stained cells.

In general, it is difficult to exploit the volume fraction ***Vmp***. We have applied the following approach:

$$Vmp * A = 2\pi r^2$$

with $r$ = cellular radius, $r = min\,[\delta(c_i, c_k), rav]$.

***rav*** = mean cellular radius of measured particles (cells in HE stain).
$\delta(c_i, c_k)$ = weighted distance/2 between nearest neighboring cells.

In order to rank the cell radii the nuclear size can be exploited to yield the cellular size, i.e.:

$$\Delta(ci) * rn_i + \delta(c_k) * rn_k = r_i + r_k * (rn_i + rn_k)$$

$rn_i$, $rn_k$ = nuclear radii of cell ***i, k*** (***i,k*** being nearest neighboring cells)
$r_i$, $r_k$ = cellular radius of cell ***i,k***.

The reference area $A$ is set $A = 1$, and the area of cytoplasm is adjusted to the size of the corresponding nuclei. In practice, the average cellular size per reference volume has to be

estimated in HE-stained slides, and an appropriate neighborhood condition has to be chosen. According to our experiences, the construction of the minimum spanning tree (MST) is the most efficient technique to select the nearest neighboring cell [Kayser et al., 1993; 1994a; 1995; 1997]. To sequentially choose cell by cell and compute the corresponding nearest neighbor is another useful computation technique.

## CALCULATION OF CYTOPLASMIC STAINING INTENSITY

A similar procedure can be applied to immuno-stained cells, again using a neighborhood condition for the calculation of the above mentioned cell size, e.g. equation **(3)**. Additional features of immuno-stained cells are:

Maximum difference in staining intensity $Dm = \Delta(Icmax, Icmin)$

$Icmax$ = maximum staining intensity / per compartment (pixel) within the cell
$Icmin$ = minimum staining intensity / per compartment (pixel) within the cell

Volume percentage of stained compartments within the cell $Pvp = \Sigma\Delta(Icp, Ico)$

$Icp$ = compartment (pixel) with staining intensity within the cell and $(\Delta(Ici, Icb) > 0)$
$Ico$ = compartment (pixel) without staining intensity within the cell and $(\Delta(Ici, Icb) \leq 0)$