

# STEREOLOGY, AN UNBIASED METHODOLOGICAL APPROACH TO STUDY PLANT ANATOMY AND CYTOLOGY: PAST, PRESENT AND FUTURE

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(Received October 11, 2017; revised November 22, 2017; accepted November 26, 2017)

## ABSTRACT

This review presents an historical overview of stereological methods used for the quantitative evaluation of plant anatomical and cytological structures. It includes the origins of these methods up to the most recent developments such as the application of stereology based on 3D images. We focus especially on leaf, as the vast majority of studies of plant microscopic structure examine this organ. An overview of plant cell ultrastructure measurements as well as plant anatomical characteristics (e.g., plant tissue volume density, internal leaf surface area, number and mean size of mesophyll cells and chloroplast number), which were estimated by stereological methods most frequently, is presented. We emphasize the importance of proper sampling needed for unbiased measurements. Furthermore, we mention other methods used for plant morphometric studies and briefly discuss their relevance, precision, unbiasedness and efficiency in comparison with unbiased stereology. Finally, we discuss reasons for the sparse use of stereology in plant anatomy and consider the future of stereology in plant research.

Keywords: chloroplast, confocal microscopy, leaf anatomy, mesophyll, stereological methods, systematic uniform random sampling

## INTRODUCTION

Plant anatomical and cytological structure has been studied since the introduction of the first magnifying devices in the 16th century. By the end of the 19th century, a number of basic developmental and functional concepts in plant anatomy were well understood (Eames and MacDaniels, 1925). For example, Julius von Sachs (1834-1897) proposed the first physiological classification of plant tissues based on their origin from uniform meristem. While some quantitative methods for studying physiological processes in plant organisms were being established, these early findings were largely based on descriptive analyses of plant anatomical structure. There was a clear need for quantitative evaluation of plant anatomy for studying relations between the function and structure of the plant body. Thus, plant scientists began to quantitatively assess anatomical characteristics, initially by morphometric, intuitive methods, and later by more rigorous approaches made possible by stereological methods.

This review presents the history of stereology applied in plant studies and discusses other methods for measuring plant structural parameters. Particular focus is given to the evaluation of leaf structure as the leaf is the most frequently studied plant organ. This is due to the fact that the specific leaf tissues, cells, and organelles (Figs. 1, 2, 8) are involved in the transfer of carbon dioxide during the process of light capture and photosynthesis, which has crucial importance for the function of the plant organism. Quantitative analysis of changes in the leaves can be very helpful in many applications studying the effect of environmental factors on plants, such as analysis of the effect of air pollution (Albrechtová *et al.*, 2007), elevated CO<sub>2</sub> concentration (Lhotáková *et al.*, 2012) or temperature (Juurola *et al.*, 2005). Moreover, leaf geometrical characteristics may be useful for interpreting physiological measurements, three-dimensional (3D) modelling during photosynthesis (Juurola *et al.*, 2005) and for phenotyping (Flood *et al.*, 2016).

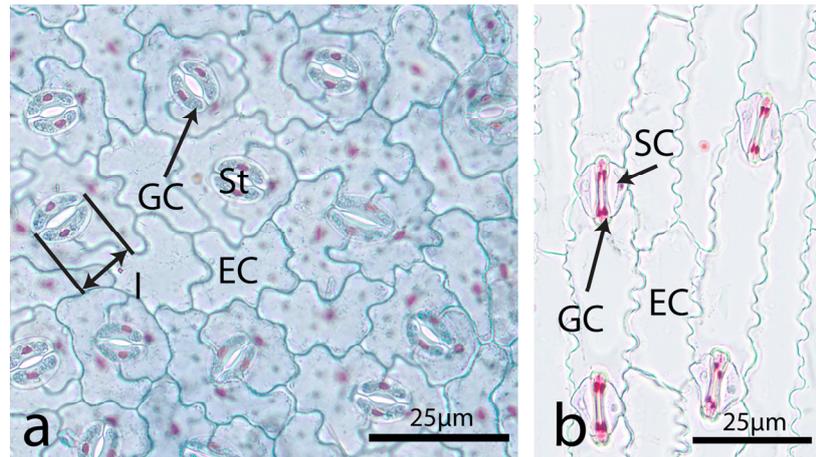


Fig. 1. Leaf epidermis. (a) *Oregano* (*Origanum* spp.) - dicotyledonous plant. St – stoma composed of two guard cells (GC), EC – epidermal cell, l – stoma length. (b) *Maize* (*Zea mays* L.) - monocotyledonous plant. Stomatal apparatus: two dumbbell-shaped guard cells (GC) and two subsidiary cells (SC), EC – epidermal cell.

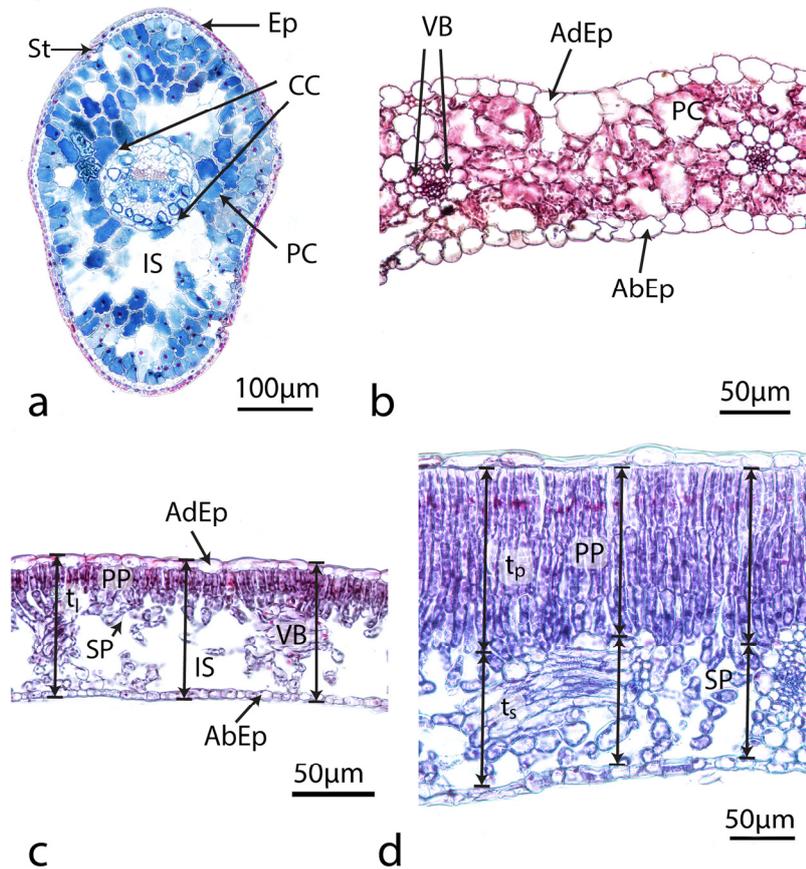


Fig. 2. Leaf internal structure in cross sections. (a) *Norway spruce needle* (*Picea abies* L. Karst): Ep – epidermis, St – stoma, CC – central cylinder with vascular tissues (round structure between arrows), mesophyll composed of parenchyma cells (PC) and intercellular spaces (IS). (b) *Monocotyledonous leaf* (barley, *Hordeum vulgare* L.): AdEp – adaxial epidermis, AbEp – abaxial epidermis, VB – vascular bundle, PC – mesophyll parenchyma cell. (c, d) *Dorsiventral leaf* (apple tree, *Malus* spp.): AdEp – adaxial epidermis, AbEp – abaxial epidermis, VB – vascular bundle, mesophyll composed of palisade parenchyma (PP), spongy parenchyma (SP), and intercellular spaces (IS). *Shade leaf* (c) with one layer of palisade parenchyma and *sun leaf* (d) with three layers of palisade parenchyma.  $t_1$  – leaf thickness,  $t_p$  – palisade parenchyma thickness,  $t_s$  – spongy parenchyma thickness.

## SHORT HISTORY OF QUANTIFICATION OF PLANT ANATOMICAL STRUCTURE

The earliest quantitative data characterizing plant anatomy were published by Friedrich Wilhelm Heinrich Alexander von Humboldt already in 1786 who presented data on stomatal density (*i.e.*, number of stomata per unit leaf area, see Fig. 1; Pazourek, 1988). Since then, many plant anatomists have used quantitative methods in their research, quite often in connection with physiological (particularly ecophysiological) and taxonomic studies, as summarized in reviews by Nátr (1988) and Pazourek (1988).

In the history of the development of quantitative approaches to investigating plant anatomy, the work of Salisbury (1928) is of particular note. Salisbury introduced the application of statistical methods in the analysis of quantitative data. He focused on determining stomatal distribution on the leaf surface and tried to prove the relations between various stomatal characteristics. He introduced the concept of stomatal index - the percentage of the number of stomata per unit leaf area with respect to the number of epidermal cells including stomata in the same unit area. A key finding of this study was determining stomatal index as less variable than stomatal density (*i.e.*, number of stomata per unit leaf area) within a particular plant species.

In general, stomatal density and stoma length in the leaves were found to be the most frequently studied plant anatomical parameters. Stoma length is usually evaluated from epidermal peels or imprints, *i.e.* from practically two-dimensional (2D) structures (Fig. 1). Leaf thickness or thickness of its tissue layers is another frequently reported parameter. It is usually measured in 2D leaf cross-sections (Fig. 2c). The thickness of tissue layers was often measured in dorsiventral leaves (Turell, 1936; Wylie, 1949). This parameter can be useful when studying important physiological phenomena associated with irradiance-affected leaf morphogenesis, *e.g.*, differentiation of dorsiventral leaves into thicker and denser sun leaves with more layers of palisade mesophyll parenchyma in comparison with shade leaf ecotypes (Fig. 2 c, d). In other plant organs, the most frequently measured parameter was the length of the root system. The root length in 2D was usually measured by the line-intercept method (Newmann, 1966), the principle of which had already been proposed by Buffon in 1777.

In the studies of internal leaf structure (Fig. 2), the physiologically important characteristics most often measured were the number and dimensions of

mesophyll cells, internal leaf surface area (*i.e.*, the surface area of mesophyll cell walls adjoining the intercellular spaces), and the proportion of intercellular spaces. In one of the first comprehensive studies of leaf mesophyll, Meyer (1923) measured the dimensions of mesophyll palisade cells - the ratio of their length to the width, variations in the length of palisade cells within a blade, the number of palisade cell layers, the angle of orientation of palisade cells to the leaf surface, and the arm palisade cells surface area. The following methods for measurement of mesophyll parameters were most frequently used: Mesophyll cell dimensions were usually measured by applying a model-based approach, *i.e.*, assuming that the cells could be modelled by simple geometrical bodies (Maksymowych, 1963; Chonan, 1966; 1970). The number of mesophyll cells was most frequently obtained by counting isolated cells after maceration (Maksymowych, 1959). Regarding internal leaf surface area, Turell (1934) also applied a model-based approach. He emphasized the importance of this parameter for photosynthetic performance, since the gas exchange is via exposed mesophyll cell walls. Furthermore, Nius (1931) examined the physiological importance of the relative volume of intercellular spaces using the infiltration method according to Unger (1854).

Following the development of electron microscopy, the first quantitative measurements of chloroplast ultrastructure emerged in the 1960s. Parameters such as the number of grana per chloroplast or thylakoids per granum were measured and 3D models of chloroplasts were introduced (Wehrmeyer and Röbbelen, 1965; Paolillo and Falk, 1966). Stereological methods were increasingly applied in the following decades, including estimating area and/or volume densities of different chloroplast compartments (Gamalei and Kulikov, 1978; Fagerberg, 1983; Kutík *et al.*, 1984).

After the establishment of stereology as a new scientific discipline in the 1960s and 70s (Weibel, 1979; Howard and Reed, 1998), design-based stereological methods emerged and began to be used also in quantitative studies of plant structures. The first applications of stereology in plant anatomy were published by Pazourek (1975; 1977), Chabot and Chabot (1977), Parkhurst (1982), Morris and Thain (1983), and Hajibagheri *et al.* (1984). Later, a number of design-based stereological methods for the estimation of various leaf characteristics were introduced by Kubínová (1987; 1989b; 1991; 1993; 1994). Characteristics highlighted included mesophyll volume, proportion of intercellular spaces in mesophyll, internal leaf surface area, mesophyll cell number and mean mesophyll cell volume, based on measurements in 2D leaf sections. Further-

more, a specific approach to root length estimation using the stereological method of total vertical projections (Cruz-Orive and Howard, 1991) was presented by Albrechtová *et al.* (1998).

Although stereological methods proposed for the estimation of leaf characteristics from 2D sections were known to be reliable, they have rarely been applied. This is mainly due to their laborious nature. Therefore, there was a clear need for less demanding, efficient and unbiased methods for measuring physiologically important mesophyll anatomical characteristics, such as internal leaf surface area, mean mesophyll cell volume and cell number in the leaf. Stereological methods based on 3D images acquired by confocal microscopy, electron tomography and other modalities, are suitable for this purpose since they are efficient and unbiased. Moreover, confocal microscopy can be applied to thick fresh plant tissue sections, thus minimizing the time spent on tissue specimen preparation and avoiding deformation of tissues due to fixation and embedding procedures.

Stereology based on 3D confocal images, called confocal stereology (for reviews see Peterson, 1999; Kubínová *et al.*, 2002; 2004, 2005; Kubínová and Janáček, 2015) is a contemporary approach that evaluates structures using a combination of stereological methods and confocal microscopy (Pawley, 1995; 2006) enabling the obtainment of perfectly registered stacks of thin serial optical sections (approx. 350 nm thick) within thick specimens. Digital images of such stacks represent 3D image data suitable for quantitative measurements. Howard *et al.* (1985) presented the first application of confocal microscopy to stereological measurements in their concept of an unbiased sampling brick. Confocal microscopy proved to be useful especially in the application of stereological methods based on spatial estimators evaluating small 3D samples of structures (Howard *et al.*, 1985; Howard and Sandau, 1992; Kubínová and Janáček, 1998; Kubínová *et al.*, 1999; Kubínová *et al.*, 2002). A 3D sample of examined tissue can be analysed if a rectangle within the microscope's field of view is focused through. Using specialized software, different virtual test probes with an arbitrary pre-defined (*e.g.*, random) position and orientation can be generated within the stack of sections and can be applied directly to this 3D image data. Albrechtová *et al.* (2007) presented the confocal stereological methods used to evaluate the mesophyll structure of narrow leaves, such as conifer needles. Kubínová *et al.* (2014) showed the application of confocal microscopy for counting chloroplasts in a mesophyll cell using optical disector principle (see below).

## OVERVIEW OF STEREOLOGICAL METHODS APPLIED IN PLANT ANATOMY

Many of the stereological methods developed over the past fifty years can be applied (directly or after suitable modification) in studies of plant anatomical structures. In this review, we present the most frequently measured plant anatomical characteristics estimated by stereological methods.

Firstly, let us mention specific features of plant anatomical structures and relevant consequences: Plant organs and cells exhibit highly variable morphology with significant differences in dimension and shape. Anisotropy (arrangement with preferential orientation) is often observed in plant tissues as well as inhomogeneity, *e.g.*, gradient in stomatal frequency in different parts of leaves, as shown by Slavík (1963) and Pazourek (1966; 1969). Gradients in tissue proportions within a leaf (Pazourek, 1977) and in mesophyll cell size along the leaf (Kubínová, 1989a) were also observed. Therefore, design-based stereological methods, yielding unbiased results without placing any assumptions on shape and arrangement of structures, are especially useful in quantitative plant anatomy.

The correct application of design-based stereological methods is critically dependent on proper sampling of tissue blocks, sections, test frames, point grids, etc. In stereology, geometrical properties of the object (in this case, the leaf) are derived from the information collected from relatively small parts of the object (*i.e.*, leaf sections). Therefore, when evaluating a specific parameter of the object (*e.g.*, the proportion of mesophyll in the leaf), just its specific parts (*e.g.*, leaf sections) are measured to estimate the parameter. In order to obtain reasonable results, these parts should be sampled in a way ensuring the estimate is close enough to the true parameter value and yielding no systematic bias. This can be achieved by proper sampling, examples of which are presented below.

### SAMPLING

#### Systematic uniform random sampling (SURS)

SURS ensures an efficient and convenient way of unbiased sampling. It has been used in the application of many stereological methods in plant research, such as the Cavalieri principle, point-counting, vertical sections, or disector methods. In leaf investigations, it can be applied for sampling leaf segments, 2D sections (Fig. 3, 4) and (3D) thick slices used in confocal stereology (Fig. 5).

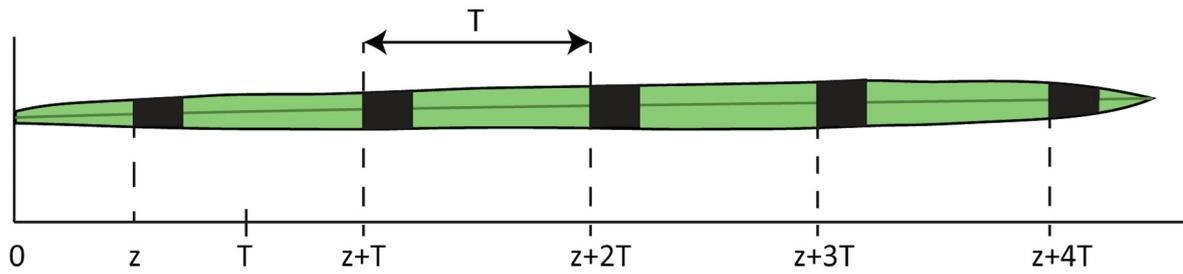


Fig. 3. Systematic uniform random sampling of segments and sections in a grass leaf. Firstly, the distance ( $T$ ) (mm) between two consecutive sections is chosen. For the position ( $z$ ), a random number is then selected from the set  $\{0, 1, \dots, T-1\}$ . The transverse sections are made in the positions  $z, z + T, z + 2T, \dots$ . For example, if  $T = 40$  mm,  $z = 20$  mm, and the leaf length is 200 mm, then the transverse sections would be cut at distances of 20 mm, 60 mm, 100 mm, 140 mm and 180 mm from the leaf base. (After Kubínová, 1993.)

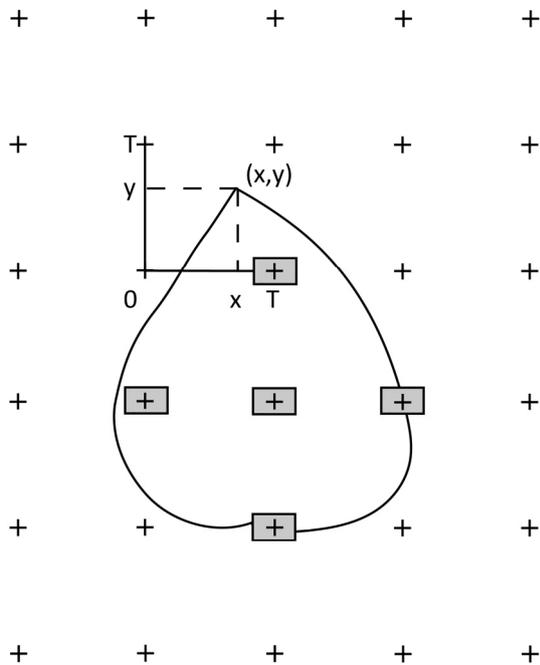


Fig. 4. Systematic uniform random sampling of segments and sections in a flat bifacial leaf. The distance ( $T$ ) (mm) between the central points of the leaf segments is chosen first. For the position ( $x, y$ ), two numbers are then (independently) selected at random from the set  $\{0, 1, \dots, T-1\}$ . By placing the leaf tip in the position ( $x, y$ ), the uniform random position of the grid of central points is ensured. The leaf segments are then cut as indicated in the figure and cross-sections are cut in the middle of the segments. (After Kubínová, 1993.)

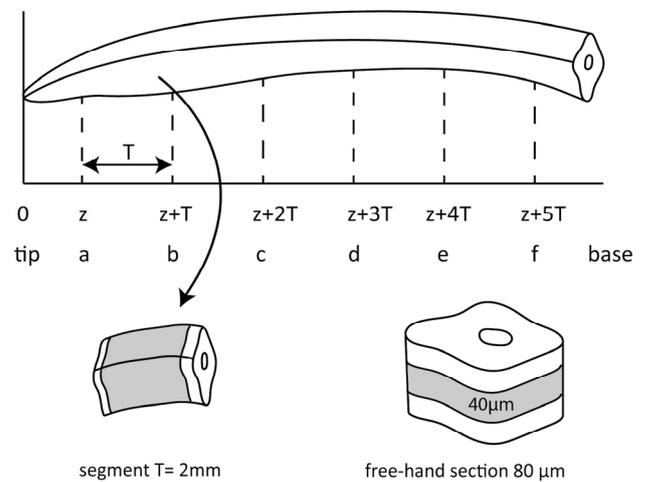


Fig. 5. Sampling design of needle specimen preparation. Upper: Systematic uniform random sampling of transverse free-hand sections:  $z =$  random position of the first section within  $(0; T]$ . The distance  $T$  between free-hand sections is chosen first. In this specific case,  $T = 2$  mm. Positions of transverse sections along the needle longitudinal axis are denoted by a, b, c, d, e, f. Lower: Left: 2-mm-thick needle segment. Right: 80- $\mu$ m-thick free-hand section from which the 40 $\mu$ m thick stack of optical sections is acquired by confocal microscopy. (After Lhotáková et al., 2008.)

### Vertical uniform random sampling (VURS)

VURS is applied in the vertical sections method (Baddeley et al., 1986). It enables the estimation of internal leaf surface area, which is one of the physiologically most important plant anatomical parameters. The practical application of VURS for narrow leaves is shown in Fig. 6.

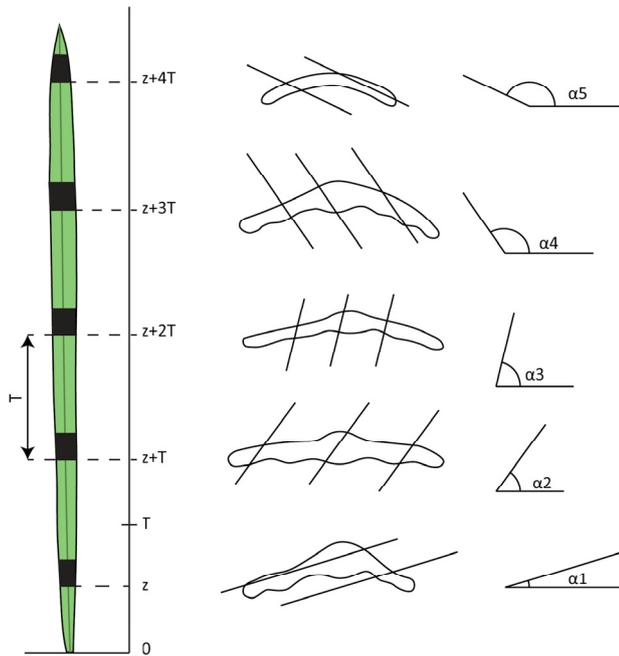


Fig. 6. Construction of vertical sections of the grass leaf. Firstly, the systematic uniform random sampling of leaf segments is done as shown on the left (see also Fig. 3). The direction of the vertical axis is chosen to be parallel to the main axis of the leaf, i.e. the vertical sections are cut in parallel with the leaf axis. At the same time they are cut around the leaf axis by an angle generated in the horizontal plane (which is perpendicular to the vertical plane) as illustrated in the figure: In the horizontal plane of the first segment (i.e. the nearest one to the leaf base; the face of the segment is shown as the lowest one in the figure), angle  $\alpha_1$  ( $0^\circ < \alpha_1 < 180^\circ$ ) is selected uniformly at random (e.g.,  $\alpha_1$  is a random number from the set  $\{0^\circ, 10^\circ, 20^\circ, \dots, 170^\circ\}$ ). The vertical sections of the first segment are cut in this direction. With  $m$  segments in the leaf, the direction of the vertical section in the  $j$ -th segment is given by the angle  $\alpha_j = \alpha_1 + (j-1) * (180^\circ/m)$  ( $j=1, \dots, m$ ). (For example, if  $m = 5$  and  $\alpha_1 = 10^\circ$ , then  $\alpha_2 = 10^\circ + 1 * (180^\circ/5) = 46^\circ$ ,  $\alpha_3 = 82^\circ$ ,  $\alpha_4 = 118^\circ$ , and  $\alpha_5 = 154^\circ$ .) Within the segment, the series of equidistant parallel sections (illustrated by lines intersecting the faces of sampled segments seen from above) are cut in a way analogous to the one described in Fig. 3. (After Kubínová, 1993.)

### Unbiased sampling of particles by disector principle

The unbiased counting or sampling of three-dimensional particles can be achieved by using the stereological method called disector principle (Sterio, 1984; Gundersen, 1986). The disector is a 3D probe which samples particles with a uniform probability in 3D

space, irrespective of their size and shape, as shown in Fig 7.

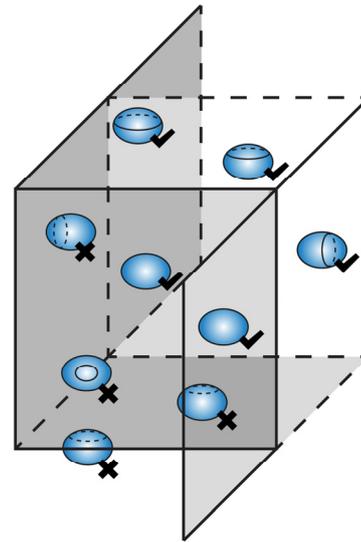


Fig. 7. Disector. Particles that are inside the 3D disector probe are counted and particles intersecting its planes are also counted except those intersecting the exclusion planes (in grey). In this example, 5 particles are counted (ticked) and 4 particles are not counted (crossed). After Kubínová et al. (2014).

## PLANT ANATOMICAL CHARACTERISTICS ESTIMATED BY STEREOLOGICAL METHODS

### Plant tissue volume density

The volume density of a specific tissue in the leaf is usually estimated by the ratio of the area of the tissue section to the area of the leaf section. The corresponding areas were measured by a point-counting method, based on counting points of the test grid falling in the tissue under study (Chabot and Chabot, 1977; Pazourek, 1977; Pazourek and Nátr, 1981; Parkhurst, 1982; Hajibagheri et al., 1984; Gowland et al., 1987; Pazourek et al., 1987; Kubínová, 1991; Albrechtová, 1994), by a planimeter (Turrell, 1936), by cutting out the enlarged drawings of the sections and weighing them (El-Sharkawy and Hesketh, 1965; Charles-Edwards et al., 1972; Dengler and MacKay, 1975), by a semiautomatic image analyser (Parker and Ford, 1982) or by a stereological method based on length measurements (linear integration method, Weibel, 1979; Thain, 1983). A number of studies (e.g., Gundersen and Jensen, 1987) showed that, in general, the point-counting method using a regular grid of points (which is positioned uniformly at random on the section) is the most effective one. Its efficiency is low only if the structure is periodic with

the same periodicity as the point grid. This can be avoided simply by changing the distances between the grid points.

The application of a point counting method for the estimation of the proportion of intercellular spaces in barley leaf was presented by Kubínová (1989b, 1991). Using this approach, the volume density of individual tissues in a leaf was measured by Edwards *et al.* (1999), Klich (2000), Luković *et al.* (2001), Bray and Reid (2002), Luković (2006), Marin *et al.* (2006), Zorić *et al.* (2011; 2014), Moura and Alves (2014), Bernardo *et al.* (2017), and Bertel *et al.* (2017). The proportion of intercellular spaces in mesophyll was also investigated by Albrechtová and Kubínová (1991), Kukkola *et al.* (2005), Albrechtová *et al.* (2007), Lhotáková *et al.* (2008), Yiotis and Psaras (2011), and Zorić *et al.* (2011; 2014). The proportion of intercellular spaces in the palisade parenchyma was measured by Konoplyova *et al.* (2008) from micrographs of paradermal sections of the leaves. Furthermore, volume density of needle tissues (Albrechtová *et al.*, 2007; Lhotáková *et al.*, 2012), trichomes in the leaf (Marin *et al.*, 2008), and petiole tissues (Luković *et al.*, 2016) were estimated.

The Cavalieri principle, which is based on multiplying the sum of areas of SURS sections by the distance between the subsequent sections (Gundersen and Jensen, 1987), was used for estimating volume of leaf and/or leaf components by Kubínová (1989b, 1991, 1993), Albrechtová and Kubínová (1991), Edwards *et al.* (1999), Klich (2000), Marin *et al.* (2008), and Albrechtová *et al.* (2007). Detailed instructions on how to apply this method are given in the overview of stereological methods for the measurement of leaf characteristics (Kubínová, 1993).

#### Surface area of mesophyll cells and internal leaf surface area

An unbiased stereological method of vertical sections (Baddeley *et al.*, 1986) was applied for the estimation of internal leaf surface area (*i.e.*, the surface area of mesophyll cell walls adjoining the intercellular spaces) and surface area of mesophyll cells (*i.e.*, the surface area of entire mesophyll cell walls) by Kubínová (1991). The method is based on counting the intersections of a special cycloidal test system with the measured surface on 2D sections generated by using VURS (Fig. 6). For more details and practical application in both narrow and broad leaves see Kubínová (1993).

Another approach was presented by Kubínová and Janáček (1998) showing application of their fakir

method for the estimation of the internal surface area of a barley leaf, and later by Albrechtová *et al.* (2007) and Lhotáková *et al.* (2008) for measuring the internal surface area of a conifer needle using confocal microscopy. Unlike the vertical sections method, which is applied to 2D physical sections, the fakir method does not require randomizing section orientation; hence the physical thick sections can be cut in any arbitrary direction. Therefore, the slices were cut perpendicular to the main axis of the needle, which is most suitable from a technical point of view (Fig. 6). The fakir method was used also by Lhotáková *et al.* (2012).

#### Number of mesophyll cells and chloroplasts

Unbiased counting or sampling of three-dimensional particles can be achieved by using the stereological method of disector (Sterio, 1984; Gundersen, 1986); for its principle see Fig. 7. Application of the disector method for estimation of mesophyll cell density and total number of mesophyll cells in a leaf was introduced by Kubínová (1989a,b; 1991). Detailed instructions on how to apply this method using SURS are given in her overview of stereological methods for estimating the number and sizes of stomata and mesophyll cells (Kubínová, 1994). The disector method for counting mesophyll cells was also used by Albrechtová and Kubínová (1991) and in combination with confocal microscopy by Kubínová and Janáček (2001), Kubínová *et al.* (2002; 2005) and Albrechtová *et al.* (2007). Kubínová *et al.* (2014) also used confocal stereology introducing the application of the disector method for estimation of chloroplast number per mesophyll cell in Norway spruce needles and comparing this approach with other methods for chloroplast counting.

#### Mean volume and surface area of mesophyll cells

Mean volume and/or surface area of mesophyll cells is estimated simply by the ratio of the mesophyll cell volume, resp. surface area, and the mesophyll cell number (for the measurement of these characteristics see above). Unbiased stereological methods were used for estimation of these characteristics by Kubínová (1989a,b; 1991; 1994; 1998), Albrechtová and Kubínová (1991) and Albrechtová *et al.* (2007).

## PLANT CELL ULTRASTRUCTURE MEASUREMENTS

Plant cell ultrastructure is often studied by transmission electron microscopy (TEM). Qualitative asses-

sment of changes in chloroplasts (Fig. 8) or other cell components is common. Most studies focused on the accumulation of starch and/or plastoglobuli, formation of lipid bodies, fragmentation of vacuoles, condensation of cytoplasm, thylakoid swelling or electron density of stroma. In some studies, a system of classes is used to describe the severity of cellular injury (Wulff *et al.*, 1996; Kivimäenpää *et al.*, 2003).

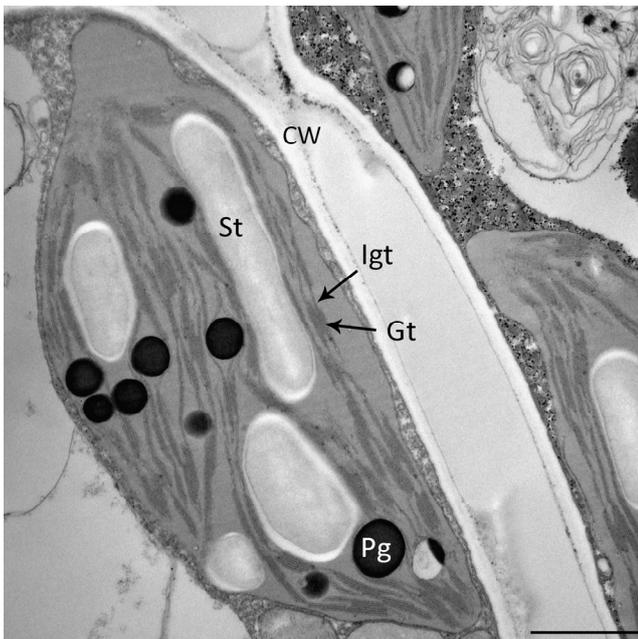


Fig. 8. *Chloroplast ultrastructure. Transmission electron micrograph of chloroplast cross-section from leaf of European beech; Gt – granal thylakoids, Igt – intergranal thylakoids, St – starch, Pg – plastoglobulus, CW – cell wall. Bar = 1  $\mu$ m.*

It should be stated that although leaf ultrastructure was sometimes evaluated by stereological methods, the application of these methods was usually done without SURS at the leaf blade level. Due to the costs and time-consuming work associated with sample preparation for transmission electron microscopy, samples for analysis were taken from the middle part or the middle third of the leaf blade or needle (in the case of conifers). Random sampling within the leaf was sometimes applied but in many studies the description of sampling design was not given in detail. In conifers, samples were taken from the middle part of the needle cross-section or from the first layer of mesophyll. Furthermore, differences in ultrastructure between the outer and inner parts of mesophyll were evaluated, mainly in studies describing the effect of stress treatment (Kivimäenpää *et al.*, 2003; Kivimäenpää *et al.*, 2014). In bifacial leaves, the ultrastructure of palisade parenchyma cells is of greater interest than spongy parenchyma cells.

For stereological analysis, the volume density of a specific part of the cell (*e.g.*, chloroplast, mitochondria, peroxisome) was estimated by the ratio of the area of the cell part to the area of the whole cell. Volume densities of thylakoids, starch, plastoglobuli and stroma inside chloroplasts were estimated by measuring the corresponding areas using the point-counting method (Kutík *et al.*, 1995; Miroslavov *et al.*, 1996; Fagerberg and Bornman, 1997; Razem and Davis, 1999; Vassilyev, 2000; Wheeler and Fagerberg, 2000; Griffin *et al.*, 2001; Gabarayeva and Grigorjeva, 2002; Pechová *et al.*, 2003; Vičánková and Kutík, 2005; Gregoriou *et al.*, 2007; Kubínová and Kutík, 2007; Holá *et al.*, 2008, Mašková *et al.* 2017).

Volume densities were usually measured on randomly sampled images of cells or organelles. Their volumes were determined only in some of the studies: Fagerberg and Bornman (1997) and Wheeler and Fagerberg (2000) used the standard leaf volume for calculation of organelle volume; Vassilyev (2000) assumed ellipsoid form of different organelles (plastids, mitochondria, peroxisomes, and dictyosomes) and calculated their volumes from their lengths and widths.

For measurements of surface densities or surface area of chloroplasts, a Merz curvilinear grid with semi-circles in a square grid was used by Fagerberg and Bornman (1997). The method of „local vertical windows“ suggested by Baddeley *et al.* (1986) was applied by Kubínová and Kutík (2007) for estimation of surface densities of thylakoid membranes. Albertsson and Andreasson (2004) estimated surface area of thylakoid membranes by drawing parallel lines across the chloroplast micrograph (perpendicular to the long axis of the chloroplast) and counting the number of membrane transections using the fact that the membrane length in randomly cut chloroplast sections is statistically proportional to the surface area of the membrane. A similar approach was also chosen by Gao *et al.* (2006).

## DISCUSSION

### OTHER MORPHOMETRIC METHODS IN PLANT ANATOMY IN COMPARISON WITH STEREOLOGICAL METHODS

In order to obtain a complex, full overview of the quantification of plant structures, it should be pointed out that many approaches other than stereological methods have been used in the past and are still being used today. However, compared to stereological methods, most methods as listed below provide biased results, most often due to improper sampling

and model-based design application. Those methods regarding their relevance, precision, unbiasedness and efficiency will be discussed below.

### Plant tissue volume density

Many authors did not measure the volume density of plant tissues in the entire leaf, but rather their tissue proportions in individual 2D sections located in non-randomized specific positions of the leaf, *e.g.*, only in the middle of the leaf (Soper and Mitchell, 1956; Sant, 1969; Charles-Edwards *et al.*, 1972; 1974; Parker and Ford, 1982). Some used specialised image analysis software for this measurement (Niinemets, 2007; Lukjanova *et al.*, 2013). However, this approach does not involve proper representative sampling, and thus it does not yield unbiased information about the proportion of tissues in the entire 3D organ.

The volume of intercellular spaces is often measured by the infiltration method (Unger, 1854; Nius, 1931; Czerski, 1968; Morrod, 1974; Byott, 1976; Smith and Heurer, 1981; Eleftheriou, 1987). Its drawbacks were discussed by Smith and Heurer (1981) and by Morris and Thain (1983). In short, the volume of intercellular spaces can be overestimated if the substance used for the infiltration enters the cells in addition to filling the intercellular spaces (Smith and Heurer, 1981), or it can be underestimated if tissue infiltration is incomplete (Morris and Thain, 1983). On the other hand, the estimation of the volume of intercellular spaces in the leaf by methods based on the evaluation of leaf section preparations can be affected by the microtechnical processing or by the section thickness effect.

### Surface area of mesophyll cells and internal leaf surface area

Many different methods have been developed for the measurement of the internal leaf surface area (sometimes also called „exposed surface area of mesophyll“). A particularly laborious procedure based on the evaluation of several transverse and paradermal sections of the leaf was proposed by Turrell (1936). The palisade and spongy mesophyll were treated separately and a kind of model shape of cells was assumed in both cases. The formula for the palisade mesophyll holds, for example, if the surface of all palisade cells exposed to the intercellular spaces are represented by the surface of cylinders (without their bases) having the same height and axes exactly perpendicular to the leaf surface. Any inclination, swelling or wrinkling of cell walls causes underestimation of the surface area of palisade cells. Similarly, the procedure described for the spongy parenchyma can lead to the underesti-

mation or in some cases even to the overestimation of its surface area.

A less laborious, but similarly biased method was used by Dornhoff and Shibles (1976) for different tissue layers of a soybean leaf (*Glycine max* L.). The exposed surface area of different mesophyll layers was estimated by the product of the thickness of the tissue layer and the total length of the trace of exposed cell walls, obtained from paradermal sections. This procedure resulted in the underestimation of the exposed surface area, because it again assumes that the orientation of cell walls is exactly perpendicular to the leaf surface and that the walls are not curved.

This method for the measurement of the surface area of mesophyll cells, also used by Dengler and MacKay (1975) and later by other authors (Parker and Ford, 1982; Barbour and Farquhar, 2004), was criticized and modified by Thain (1983), who proposed several curvature correction factors eliminating the error caused by the curvature of cell surfaces, assuming different model shapes of mesophyll cells. Some of these mesophyll cell model shapes were more realistic than cylinders with hemispheres on each end (assumed by Nobel *et al.*, 1975) or spheres (Bunce *et al.*, 1977).

Thain's method (1983) was used also by Longstreth *et al.* (1985), Miyazawa and Terashima (2001) and Oguchi *et al.* (2003). Sasahara considered the palisade mesophyll cells of *Brassicaceae* leaves as special solids of 'intermediate shapes between spheroids and columns' (Sasahara, 1971) and the mesophyll cells of *Triticum* as systems of interconnected cylinders with hemispheres on each end (Sasahara, 1982). A similar model of the *Triticum* mesophyll cell was used by Chonan (1965). It should be stressed here that it is always necessary to verify the appropriateness of the model for each type of structure under study, which may be difficult and laborious.

The stereological method, based on counting intersection points between the studied surface and line probes, has also been used for the estimation of the internal leaf surface area per unit leaf volume or the surface area of mesophyll cells per unit leaf volume. However, certain model assumptions were always considered. Morris and Thain (1983) claim that they achieved isotropic orientation of cell walls (*i.e.*, with no preferred orientation) by preparing a suspension of the isolated mesophyll cells, which were then embedded and cut. The isotropic structure of the spongy mesophyll of tobacco (*Nicotiana tabacum* L.) leaf was assumed (and verified) by Gowland *et al.* (1987) who considered the palisade

mesophyll as a partially orientated linear system of surfaces.

The stereological method based on counting intersections was also used by Parkhurst (1982) for the estimation of internal leaf surface area. Special models of mesophyll cells (cylindrical type, isotropic type, and a rather controversial intermediate type of structure) were assumed. Some of the pitfalls of Parkhurst's approach (disregarding the end surface of cylinder-like cells, subjective estimation of the degree of 'cylindricity' of the tissue) were discussed by Thain (1983). James *et al.* (1999) also used a model-based approach to the measurement of the mesophyll surface area per leaf area from oblique-paradermal sections – the latter exploited Image-Pro Plus software. Slaton and Smith (2002), Khramtsova *et al.* (2003), and Rhizoupoulou and Psaras (2003) also measured this parameter by model-based methods.

In summary, all the above mentioned methods for the estimation of surface area of mesophyll cells and internal leaf surface area were based on the choice of a specific, more or less realistic model of mesophyll cells. This model-based approach, when applied to real biological objects, such as mesophyll cells, brings about a bias which is challenging to quantify. Moreover, this approach cannot be reasonably used for the measurement of structures which cannot be approximated by simple geometrical models. This is the case for the mesophyll of grass leaves and coniferous needles where the cells have an irregularly lobed shape and possess a certain degree of anisotropy. Recently, Theroux-Rancourt *et al.* (2017) have tested several model-based methods for mesophyll surface area estimation. They found that the model-based methods often led to underestimation of this parameter (up to 30%) in comparison with 3D method based on the evaluation of 3D image data acquired by microCT using specialized ImageJ plugins. However, they have not tested stereological methods, since “these methods have been less adopted in recent years”.

Unbiased stereological methods can indeed avoid the above mentioned problems. They are using the design-based approach, where uniform random position and random orientation of line probes must be ensured, as is the case for the methods of vertical sections (Kubínová, 1993) and fakir method (Kubínová and Janáček, 1998) described above. This approach is precise and more efficient (especially the fakir method in combination with confocal microscopy as shown by Albrechtová *et al.*, 2007) than model-based methods. Its advantages have been acknowledged by other authors (El-Sharkawy, 2009).

## Number of mesophyll cells and chloroplasts

The number of mesophyll cells was usually determined by counting isolated cells after their maceration (Maksymowych, 1959; 1963; Smith, 1970; Morrod, 1974; Jellings and Leech, 1984; Sasahara, 1982; Lieckfeldt, 1989). This method may lead to the loss of cells during manipulation of the cell suspension or by damage to certain cells and, accordingly, to the underestimation of the cell number. Wilson and Cooper (1967) and Adachi *et al.* (2013) applied a 2D approach where they counted cell profiles while making assumptions about cell shapes, potentially leading to biased results. Using the stereological method of optical disector gives a theoretically unbiased result. However, in practice it is necessary to fulfil the so called General Requirement, *i.e.*, each cell has to be unambiguously identifiable from its profiles in studied stacks of optical sections (*i.e.*, 3D image data containing a disector probe, typically acquired by widefield or confocal microscope).

One of the most frequently used methods for the estimation of chloroplast number per mesophyll cell in herbaceous plants in 2D is counting chloroplasts in separated mesophyll cells obtained by maceration procedures as it was described by Possingham and Saurer (1969). This technique was used by many authors (Possingham and Smith, 1972; Boffey *et al.*, 1979; Lamppa *et al.*, 1980; Molin *et al.*, 1982; Tymms *et al.*, 1983; Sung and Chen, 1989; Pyke and Leech, 1991; Yamasaki *et al.*, 1996; Marrison *et al.*, 1999; Ivanova and P'yankov, 2002; Meyer *et al.*, 2006; Stettler *et al.*, 2009). This estimation is unbiased since counting is done in the whole cell, provided the chloroplasts in the specimen are not overlapping and the cells used for chloroplast counting are selected in an unbiased way. However, in some plant species it is impossible to macerate separate cells, especially in coniferous needles and leaves with mesophyll cells with lignified cell walls. In this case the disector method (Fig. 7) can be efficiently used for unbiased estimation of chloroplast number per mesophyll cell using confocal microscopy (Kubínová *et al.*, 2014).

Practical and theoretical tests presented by Kubínová *et al.* (2014) demonstrated that the frequently used method for chloroplast number estimation by counting profiles of particles from 2D sections yielded biased estimates (e. g. Boffey *et al.*, 1979; Miyazawa and Terashima, 2001; Sam *et al.*, 2003; Zechmann *et al.*, 2003; Wang *et al.*, 2004; Hayashida *et al.*, 2005; Oguchi *et al.*, 2005; Teng *et al.*, 2006;

Gopi *et al.*, 2008; Maslova *et al.*, 2009; Jin *et al.*, 2011; Simon *et al.*, 2013) and that the results may be one order of magnitude different from the real chloroplast numbers. Another possible method is to count the chloroplasts in cells directly during focusing through the specimen using conventional light microscopy (Ellis and Leech, 1985; Bockers *et al.*, 1997) which can be applied if entire cells can be focused through and the chloroplasts are sparsely distributed in cells. However, chloroplasts usually tend to be densely packed along the cytoplasmic membrane. The chloroplast number per cell was also determined in 3D reconstructions made from a series of confocal microscope images (Mozafari *et al.*, 1997; Dinkins *et al.*, 2001; Coate *et al.*, 2012; Xu *et al.*, 2012). Such a method can yield an unbiased estimate if SUR sampling is applied and a sufficient number of cells is analysed. However, this is a much more time-consuming approach than direct application of the disector method in combination with confocal microscopy. In conclusion, the disector method can be applied universally and provide unbiased estimation of the number of particles.

#### Mean volume and surface area of mesophyll cells

The volume of a mesophyll cell is usually estimated by the volume of a simple geometrical body by which the cell is approximated. Palisade cells were modelled by ellipsoids (Wild and Wolf, 1980) and cylinders (Maksymowich, 1963; Morrod, 1974; Gowland *et al.*, 1987) or by cylinders with hemispherical ends (Sasahara, 1971; Barbour and Farquhar 2004; Burundukova *et al.*, 2003; Khramtsova *et al.*, 2003). Spongy cells were approximated by spheres (Morrod, 1974; Charles-Edwards *et al.*, 1974) or ellipsoids (Wild and Wolf, 1980). Chonan (1965) and Sasahara (1982) modelled lobed mesophyll cells of wheat (*Triticum* sp.) by systems of parallel cylinders with hemispherical ends (standing side-by-side).

In most cases a model-based approach was chosen. Taking into account that it is often difficult to judge the appropriateness of the model used, this approach brings about a bias which is difficult to quantify. Moreover, this approach cannot reasonably be used for the measurement of cells which cannot be approximated by simple geometrical bodies.

As in the cell volume measurements, the model-based approach to the measurement of the surface area of mesophyll cells prevailed (Chonan, 1965; Morrod, 1974; Sasahara, 1971; 1982; Bunce *et al.*, 1977; Tichá and Čatský, 1977; Ivanova and P'yankov, 2002). It should be emphasized that, in comparison

with the cell volume measurement, the estimation of the cell surface area by the surface area of a simple geometrical body can lead to even more pronounced bias since neither the inclinations, swellings nor wrinklins of the cell wall are taken into account. This can cause severe underestimation of the cell surface area.

In summary, unbiased stereological methods avoid the problems of model-based approach discussed above and can be recommended for mesophyll cell volume and surface area measurement. For this purpose, combination with confocal microscopy is especially useful (Albrechtová *et al.*, 2007).

#### OTHER METHODS FOR THE MEASUREMENT OF PLANT CELL ULTRASTRUCTURE

Plant cell ultrastructure is recently most often quantified by image analysis on digitized images of plant cells or chloroplasts acquired by transmission electron microscopy (TEM). Typical parameters measured are thickness of the cell wall, size of organelles (length, width, profile area) – mostly of chloroplasts and mitochondria, proportions (in %) and numbers of the organelles (Liu and Dengler, 1994; Lepeduš *et al.*, 2001; Oksanen *et al.*, 2001; Yu *et al.*, 2017). Inside the chloroplasts, the size, number and profile area of starch grains, plastoglobuli and granal thylakoids are measured (Wulff *et al.*, 1996; Pritchard *et al.*, 1997; Schmitt *et al.*, 1999; Bondada and Syvertsen, 2003; Riikonen *et al.*, 2003). Thylakoids are also quite frequently counted and presented as numbers of thylakoids per granum (Demmig-Adams *et al.*, 2015; Ren *et al.*, 2017). It is a well-known fact that the number of thylakoids per granum is different for plants grown in sun or shade conditions (Boardman, 1977). Unfortunately, most studies do not describe sampling design in detail and counting of particles on 2D sections of chloroplasts or cells is probably biased in a similar way as in the case of chloroplast number estimation.

Some authors realize that counting particles in 2D may not be reliable. For example, Kivimäenpää *et al.* (2014) did not count the number of mitochondria on 2D sections, because they realized that the individual mitochondrial cross-sections can be from the same, long, branched and folded organelle.

3D methods for the determination of plant cell ultrastructure are used rather sparsely. In some studies (Perktold *et al.*, 1998; Zellnig *et al.*, 2004) the method of ultrathin serial sections was used as a way to build up a 3D image of the organelles, where the volumes of the organelles and their parts could be estimated

(using Cavalieris principle). Crumpton-Taylor *et al.* (2012) used a FIB-SEM method for measurements of chloroplast and starch granule volume as well as granule numbers in *Arabidopsis*. In comparison with the serial sectioning and TEM, this method has advantages of speed and accuracy as there is no distortion of the image surface and alignment of the images is rather simple. Unfortunately, this method requires very expensive equipment. Using 3D methods, all starch granules inside the chloroplasts can be counted; any possible bias is thus limited to the possibility of incorrect sampling of small tissue samples or differential shrinkage of cell compartments during the sample preparations.

### SPARSE USE OF STEREOLOGY IN PLANT ANATOMY

Although stereology provides a number of valuable tools for unbiased and precise measurement of plant structural characteristics, it has been used in this field sparsely until now. This is illustrated by the graph in Fig. 9 showing that the gap between the number of publications on stereology in plant science and their number in animal/human biosciences has been increasing since the 1990s. (The graph is just indicative as it is clear that it does not show all publications using stereological methods.)

What can be the reason for the sparse use of stereology in plant anatomy? It may be that many plant biologists are not acquainted with stereological methods and/or find them to be too complicated and laborious. In the community of plant biologists, broader publicity should be made through educational materials such as courses, tutorials, reviews, Wiki, etc. We envisage that this can be made possible on a broader scale by collaborative efforts such as the International Society for Stereology and Image Analysis (<http://www.issia.net/>). It should be made clear to the plant biological community that stereological methods are not so difficult to apply and yield unbiased results unlike many other methods, which can lead to erroneous results and, moreover, are often also quite tedious and time consuming. More studies bringing supporting arguments and comparisons of stereological and other methods should be made. This has been done, for example, for the estimation of volume and surface area of tobacco cell chains (Kubínová *et al.*, 1999) where different stereological and image analysis methods were compared. In this case, it was concluded that the fakir method and the Cavalieri principle enable interactive, unbiased and efficient estimation of the cell surface area and volume.

It should also be noted that the application of automatic image analysis methods for measurements of plant structures is often limited by difficulties with proper automatic detection (segmentation) of these structures in microscopic images.

The popularity of stereological methods could also be increased by decreasing their laboriousness. This is possible by using specially dedicated software, either directly connected with image acquisition equipment, or stand-alone software modules. A combination with semi-automatic image analysis, while keeping proper sampling schemes, can also make measurements more efficient in some cases. Special attention should be paid also to the selection of suitable methods for each specific type of measurement and to the most efficient sampling design, as well as to all practical aspects of the measurement procedure.

Based on the above facts, we believe that stereology will find its way to the community of plant biologists and become one of the most powerful tools for investigation of plant anatomical and cytological structures.

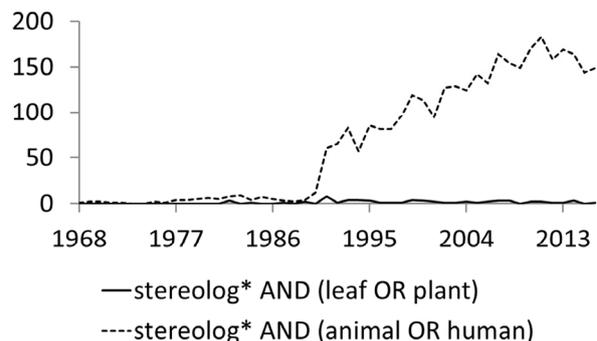


Fig. 9. Number of publications on stereology in plant and animal/human biosciences in the past 48 years, i.e., since the first publication including the key world *stereolog\** has appeared. Based on the result of search in *Web of Science* with key words (*stereolog\* AND (leaf OR plant)*) or (*stereolog\* AND (animal OR human)*), respectively.

### ACKNOWLEDGEMENTS

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic (LM2015062 Czech-BioImaging project; NPUI LO1417 project; CZ.02.1.01/0.0/0.0/16\_013/0001775 Modernization and support of research activities of the national infrastructure for biological and medical imaging Czech-BioImaging funded by OP RDE), project ERDF, OPVK BrainView CZ.2.16/3.1.00/21544, and by the project SVV of the Charles University. We

thank Drahomíra Bartáková for technical help with anatomical preparations and Miroslav Barták (both from the Faculty of Science, Charles University) for his help with photographing and figure processing.

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