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Technical Note

# A new Fiji-plugin for visualizing intra-annual density fluctuations and analyzing intra-annual theoretical volumetric flow rate fluctuations along wood cross-sections

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Intra-annual analysis of wood samples by examination of xylem structure offers the possibility to reveal structural adaptations in the xylem of trees to varying climatic conditions with a high temporal resolution. Additionally, it can help closing the gap between annual tree ring width measurements and ecophysiological field studies.

Typically, two approaches for intra-annual wood analyses are used: densitometry (x-ray or high frequency measurements) and analyses of microscopic images of cross-sections. While densitometry has recently become the most commonly used method, it requires expensive measurement devices, which are not necessarily part of standard laboratory equipment and accordingly not affordable for a significant number of researchers.

We therefore present a new plugin for visualizing intra-annual density fluctuations (IADF) and, more specifically, for analyzing the theoretical volumetric flow rate with an adapted Hagen-Poiseuille law for elliptic conduits per pixel-column. The implemented algorithm represents an alternative for intra-annual analysis of microscopic cross-section images. The plugin has been developed for Fiji, a common package based on the open-source image processing program ImageJ to avoid the use of commercial programs for anatomical analyses.



## 1. Introduction

Dendroecological analyses based on intra-annual research of tree-ring samples have gained importance over the past years as it allows for an increased temporal resolution rather than performing dendroecological analyses on a year-to-year basis. In combination with climate data with a high temporal resolution it can offer a better understanding of structural xylem fluctuations even at a seasonal level (cf. de Micco et al., 2012). Furthermore, several studies have shown that species-specific direct links exist between seasonal climatic conditions including severe drought and precipitation events throughout a year and the occurrence of certain types of intra-annual density fluctuations (IADF) inside the respective tree ring (e.g. Wimmer et al., 2000; Campelo et al., 2007; Vieira et al., 2009; de Micco et al., 2016; Zalloni et al., 2016).

To analyze intra-annual xylem structure fluctuations, different densitometric methods have been developed and adapted over the past decades. These methods include mainly X-ray densitometry (Polge, 1978), high-frequency densitometry (Schinker et al., 2003) and neutron imaging (Mannes et al., 2007). The common drawback of these methods is the need of highly specialized and hence expensive laboratory equipment. Beside these densitometric approaches, a less destructive measurement method exists for living trees by analyzing tree-ring profiles through drill resistance. While this approach is useful to determine timber quality and to detect decayed wood parts, minimal changes in the piercing angle inside the tree can lead to an unfavorable curve shift on a high-resolution intra-annual level (cf. Rinn et al., 1996). Hence, in recent times IADFs are commonly analyzed through visual identification with a stereo microscope and wood samples with a carefully polished or cut surface. While subjectivity of this method can mostly be avoided through detection of IADFs by several trained but independent operators, it still cannot be completely excluded and can highly depend on the individual sample and sample preparation quality (cf. Battipaglia et al., 2016; Zalloni et al., 2016).

Therefore, the anatomical analysis of xylem structure can offer a more quantitative and less subjective approach by automation. Furthermore, it has been shown by Decoux et al. (2004) that calculated wood-density profiles from anatomical images can be compared to densitometric measurements in close approximation. However,

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they have to be calibrated carefully with species-specific cell-wall density values (cf. Kellogg and Wangaard, 1969) to avoid differences, and additional visual identification is still mandatory (cf. Battipaglia et al., 2016).

Besides structural stability, the second main function of xylem cells is to conduct water (Schweingruber, 1996) and recent studies have strengthened the importance of variations of water availability throughout the growing season for xylem structure and intra-annual cell differentiation (Carvalho et al., 2015). Analyzing xylem hydraulic architecture as a proxy can thus reveal seasonal adaptations of a tree to variable environmental conditions (cf. Fonti et al., 2010) and in this context the lumen shape of water conducting cells is important to characterize the hydraulic conductivity (cf. Battipaglia et al., 2016). Hence, determining intra-annual theoretical volumetric flow rate fluctuation (hereinafter: IAVFF) per conduit as presented in this study is an alternative approach to determine xylem structure variations throughout a tree ring by analyzing microscopic cross-section images of high resolution.

For anatomical measurements, several specialized programs and tools already exist including e.g. WinCell (Regent Instruments Inc., Ville de Québec, Canada), AxioVision (Carl Zeiss Microscopy GmbH, Jena, Germany) and ROXAS (Wegner et al., 2013; Arx and Carrer, 2014). These programs and tools offer a large amount of options and parameters. WinCell and ROXAS are highly specialized for in-depth wood anatomical analysis, including volumetric flow-rate calculations based on the Hagen-Poiseuille law. However, the available programs and tools are mostly either embedded in commercial programs or plugins for those programs. In contrast, there are highly customizable open source programs such as ImageJ or Fiji, which are based on Java and allow implementing user-specific analysis methods through plugins.

Accordingly, we herein describe a new approach in which only standard laboratory equipment for the preparation of high-resolution microscopic images, open source and freeware programs for their analysis are needed. This approach involves the freeware program Image Composite Editor ICE (Microsoft Research, Computational Photography Group), the mentioned open source program Fiji or ImageJ 1.48o, respectively, with Java 1.6.0_24 (64 bit) and a customizable Python-plugin to analyze the before-mentioned parameters. In addition to existing programs and tools, the plugin presented here provides an adapted intra-annual theoretical volumetric flow rate analysis to record IAVFF as a pixel-column-wise parameter for ImageJ/Fiji in order to combine tree-ring-based wood anatomical analyses with ecophysiological measurements (e.g. Rzepecki et al., 2011).

## 2. Material and methods

### 2.1. An open-source approach using Image Composite Editor (ICE) and ImageJ/Fiji

ImageJ is a common open source image-processing software tool based on Java with a long history of scientific image analysis (Schneider et al., 2012). This cross-platform compatible tool enables implementing issue-adapted plugins. To this end, a large stock of macros and plugins is already available. Using ImageJ is therefore valuable for the here-proposed open-source approach for analyzing intra-annual xylem features. In order to increase the accessibility of our plugin even further, we decided to use ImageJ's Fiji distribution (Schindelin et al., 2012), which includes the option to write macros and plugins using the open-source programming language Python (Python Software Foundation, 2016). Using this cross-platform language with a focus on code readability in combination with ImageJ/Fiji built-in macro functions even ensures that the plugin and the introduced parameters are accessible and modifiable in common operating systems. Accordingly, all programming for the plugin was performed with Python and Python's Integrated Development Environment (IDLE) v. 3.4.2.

### 2.2. Sample and picture preparation

In order to provide a proof of concept for the correct operation of the plugin's algorithm we gathered stem discs from two freshly cut 50-year-old *Pseudotsuga menziesii* (Douglas fir) and *Picea abies* (spruce) trees at two adjacent monospecific stands in the "Osburger Hochwald" (49.713 N°, 6.887 E° [WGS84], Rhineland-Palatinate, Germany). Rectangular samples were separated from these stem discs including the years 1975–1979. Subsequently, continuous cross-sections (thickness ca. 20 μm) were cut with a GSL1- and a Core-microtome (Gärtner et al., 2014; Gärtner and Nievergelt, 2010) both developed at the Swiss Federal Research Institute WSL.

To increase the contrast between cell wall and lumen, samples were stained with safranin and rinsed with increasing concentrations of ethanol (40%, 60%, 90%), isopropanol and Roti®-Histol (Carl Roth GmbH, Karlsruhe, Germany). For fixation, micro-sections were subsequently embedded in Roti®-Histokitt (Carl Roth GmbH, Karlsruhe, Germany).

In a further step, overlapping RGB color space pictures along the sample were taken with a stereo microscope Axio Imager M1 and a connected digital camera AxioCam Mrc 5 (Carl Zeiss Microscopy GmbH, Jena, Germany). Each image, captured with 50x magnification, was saved in the TIFF-format to prevent any data loss. While the ongoing progress in the field of computer hardware – concerning the amount of random access memory in common work stations – enables the composition of big TIFF-image amounts problems can arise when computers with RAM below 4 gigabyte and a 32 bit operating system are used. As an alternative, if the data size of the sum of these images becomes too large, the use of low-compressed JPEGs can be considered to speed up the following stitching process but should be avoided if possible. For a simple subsequent scaling of images in Fiji, it is a useful addition to retain one image of each series with an integrated scale bar.

The high resolution single shots along the sample can then be combined by using any picture stitching program, but to prevent panorama distortion, which would have an unfavorable effect especially on the subsequent calculation of the theoretical volumetric flow rate per channel (tracheids or vessel elements), a planar stitching method has to be applied (Rzepecki et al., 2011; Dettmann et al., 2013). Following our freeware approach we used the ICE 64 bit program for high resolution (0.92 pixels $\mu m^{-1}$) sample pictures. Even for gigapixel images it offers a comparatively fast stitching process and in a previous study, we could show that there is a clear correlation between the number of conduits and the sum of all individual conduit cross-sectional areas between original images and the corresponding sections of the stitched image (Rzepecki et al., 2011). Nevertheless, any other distortion-free stitching program or even stitching plugins for ImageJ/Fiji can be used, but should be carefully documented.

Subsequently, the RGB composition picture was transformed into a binary version to make the plugin algorithm work. We used the Fiji inbuilt binary threshold function for this purpose (Fig. 1a and b), though other methods and plugins such as common Otsu (cf. Otsu, 1975) and Multi-Otsu-threshold methods (cf. Tosa, 2006) may also be used. In any case, the threshold method should be documented for subsequent traceability.

For the plugin, the received binary pictures were horizontally oriented preferably from pith to bark with vertical tree-ring borders. If the tree-ring borders are not orthogonal but inclined, we suggest separating each year by choosing a rectangular region of interest (ROI) beginning in the earlywood and adapted to the tree-