



## Research

### Evaluation of a confocal WSI scanner for FISH slide imaging and image analysis

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#### Abstract

**Background:** Technological advances contribute to the maturation of digital pathology in clinical and research applications. However, there are only few reports on fluorescence scanning especially on confocal fluorescence imaging technology in digital pathology, which has superior depth resolution compared to wide-field fluorescence imaging. Here, we explored the features of a confocal WSI scanner for typical diagnostic and research imaging applications of fluorescence in situ hybridization (FISH) assay.

**Methods:** Multi-layer stacking (Z-stack) which stores all image information from each layer, and extended focus which stores the optimal image information from all scanned layers, featured in the Panoramic Confocal scanner (3DHISTECH Ltd., Budapest, Hungary) were employed in digitizing 14 FISH slides (ALK, EGFR, and multi-gene). The slides were scanned with a 40× water immersion objective producing a final image with pixel resolution of 0.1625 µm/pixel. Z-stack and extended focus were used for N=6, 13, and 26 multiple layers scanning at 1, 0.4, and 0.2 µm depth intervals respectively. Single-layer scanning was also done for comparison. Scanning time and resultant file size were recorded. The CaseViewer from 3DHISTECH was used to visualize images and export the annotated regions, and the exported images were further analyzed in Imaris (Bitplane, Zurich, Switzerland) for 3-dimensional reconstruction, nuclear segmentation,



and the quantification and co-localization analysis of dots inside nuclei. Quantification data from Imaris were imported into Excel for statistic analysis.

**Results:** Confocal fluorescence scanning of FISH slides enabled sharper image than wide-field scanning, although it required longer scanning time and larger file storage. More intra-nuclear dots were quantified from multi-layer Z-stack images than single-layer images, and the Z-stack increased scanning time and image file size. Furthermore, there were a reduced in the number of dots and an increased in the number of co-localized dots in extended-focus images compared to Z-stack. Dots in multiple channels were quantified and analyzed automatically, which supports clinical diagnosis of gene amplification, deletion, and translocation. Three-dimensional reconstruction of Z-stack produced precise measurement of spatial distance, which supports molecular research.

**Conclusion:** Confocal provides sharper image than wide-field for FISH slide scanning. Extended focus reduces file size and storage, but could cause inaccurate analysis due to misinterpretation of overlapping information. Z-stack scanning provides high volume image information for spatial analysis. We foresee confocal multi-layer scanning as a digital pathology application tool for FISH imaging in both clinical and research in future.

**Keywords:** [Fluorescence in situ hybridization](#); [Whole slide image](#); [Confocal](#); [Three-dimensional reconstruction](#); [Image analysis](#)

## 1. Introduction

Cellular and molecular imaging technologies that permit the accurate localization of genes as well as their products within cells and tissues are in increasing demand for future precision medicine. Fluorescence in situ hybridization (FISH) is a powerful technique employed in clinical and research laboratories to visualize specific DNA/RNA sequences within the nucleus and provide the presence, location and/or structural integrity of genes on chromosomes. FISH incorporates fluorescently labeled probes that can bind specifically to the segment of the genome with which they have a high degree of sequence similarity. FISH thereby is an approach to simultaneously and accurately localize multiple signals within cells. FISH is thereby a core molecular diagnostic tool with implemented clinical application in personalized therapy and biomarker research for cancer [1-4].

Both wide-field fluorescence microscopy (epifluorescence microscopy) and confocal fluorescence microscopy are commonly used for fluorescence imaging. In wide-field microscopy,



fluorescence emission from the specimen on the focal plane as well as above and below the focal plane is gathered for final image formation. In confocal microscopy, however, fluorescence emission gathered from the specimen is filtered through a confocal pinhole aperture, which allows the microscopy to collect the emission coming from the focal plane only. Therefore, confocal fluorescence imaging has the following advantages over wide-field fluorescence imaging: (i) elimination/reduction of background information from focal plane; (ii) lower excitation energy; and (iii) the ability to perform serial optical sections with thick specimen [5, 6]. These advantages facilitate the image-based detection of mutations particularly these through the analysis of enumeration of multiple genes within nuclei and the analysis of local relationship of signals with different colors [5]. Confocal imaging technology has the unique feature of enabling optical sectioning of thick tissues, which is critical for three-dimensional (3D) tissue reconstruction for volumetric spatial analysis.

Manual image acquisition by fluorescence microscopy despite being time-consuming and subjective can be considered the standard in many laboratories. Therefore, methods for data acquisition should be standardized as well as automated so that less manual work is required from the investigator and high throughput and objectivity could be achieved. Whole slide imaging (WSI) technology has been developed to automate the digital image acquisition from glass slide. WSI is also a very promising technology for the effective implementation of decision support systems based on computer-assisted automated image analysis [7-8]. Although there are many WSI scanners designed for bright field imaging, there are very few WSI scanners that work well for FISH slide digitizing. A WSI fluorescence scanner has been reported to be rapid, robust, and highly sensitive for interpreting FISH slide of diffuse large B cells lymphoma cases with break-apart probes to detect MYC rearrangement [9]. Another two microscope and image analysis systems but not WSI scanners were reported to have the capability to digitize FISH slide for the detection of HER2 in breast cancer [10] and dual-fusion and break-apart probes in lymphoma [11]. As far as we know, there is no confocal WSI scanner reported for FISH slide imaging and analysis yet.

Multi-layer Z-stack and extended focus technologies have been developed to acquire the digital images of thick samples which have critical information in multiple focal planes [12, 13]. In multi-layer Z-stack scanning, the tissue is scanned independently at N different layers at a specified depth thereby producing N scanned images which are kept for viewing and analysis later. Since



all the N layers of images are kept, Z-stack scanning requires large data-storage space. To ensure seamless viewing experience of these images, it is important that the client computer meets the necessary hardware specifications, i.e. RAM, processor speed, graphics/video cards, etc, and network connection should allow for huge data transmission. Extended focus, on the other hand, uses an algorithm that extracts the focus areas from each scanned focal plane and assembles them to form a single composite image (Supplementary Figure 1). This ameliorates the large storage and high-speed network connectivity requirements in multi-layer Z-stack scanning. The composite image formed from this process, however, has been demonstrated to contain subtle image information that is not necessarily observed from single layer scanned images [12]. Through the volume reconstruction and rendering technologies, Z-stack scanning of FISH slide allows three-dimensional visualization of individual gene loci, sub-chromosomal domains, and even entire chromosomes, which are impossible for extended focus scanning. This advantage of Z-stack over extended focus is especially meaningful in researches that investigate the spatial organization of the genome and its function during interphase [14, 15].

A confocal WSI scanner, which can do both bright field and fluorescence scanning, was introduced by 3DHistech (Budapest, Hungary) recently. The fluorescence scanning features two modes: wide-field and confocal. In this study, we were aimed to explore this confocal WSI scanner for application in typical diagnostic and research imaging applications of FISH assay.

## 2. Materials and Methods

This study was approved by institutional review board of Massachusetts general hospital, Boston, MA, USA.

### 2.1 FISH slides

Totally of 14 FISH slides were retrieved from the hospital pathology department. Six of anaplastic lymphoma kinase (ALK) FISH slides, six of epidermal growth factor receptor (EGFR) FISH slides, and two of four-probe FISH slides were scanned. For ALK FISH slides, tissue sections were hybridized with dual color break-apart rearrangement probe (Abbott Molecular, Illinois, USA) to detect gene translation; while in EGFR FISH slides, tissue sections were hybridized with copy number probe (Abbott Molecular, Illinois, USA) to detect gene amplification. ALK, EGFR FISH tests were performed on lung cancer samples from surgical resection. All samples were



sectioned with thickness of 4  $\mu\text{m}$ . The probe information about the genes of the multi-gene FISH slides was unknown. No clinical patient data were retrieved for this study.

## 2.2 Confocal WSI scanner

In this study, FISH slides were digitized with the Panoramic confocal scanner (3DHISTECH Ltd., Budapest, Hungary) with its 40 $\times$  water immersion objective which has a numerical aperture (NA) of 1.2, thereby producing a final image with a resolution of 0.1625  $\mu\text{m}/\text{pixel}$ . The confocal scanner can be implemented with up to 9 filter sets. In the current study, five filters (DAPI, FITC, TRITC, Aqua, and Cy5) were chosen in according with their fluorescent excitation and emission wavelengths of the probes. The scanner uses the Lumencor SPECTRA 7 Light Engine (Oregon, USA) as the source of its excitation light and the CMOS pco.edge 5.5 camera (Kelheim, Germany) as the detector to capture the image signals. Its confocal unit, which includes the filter sets, is the Aurox CC88 (Abingdon, UK). The optical components of this scanner allow both bright field and fluorescence imaging. Both wide-field and confocal modes are provided for fluorescence imaging. The scanner supports fully automated scanning and semi-automated scanning. Whereas in fully automated scanning the definitions of the focus maps as well as the detection of the tissue regions are left to the control software to decide, semi-automated scanning allows user to define the focus map as well as the tissue regions to be scanned.

## 2.3 FISH slide scanning

Totally of 14 FISH slides (ALK, EGFR, and multi-gene) was scanned at both single layer and multiple layers with FITC, TRITC and DAPI filters (another two filters Aqua and Cy5 for multi-gene FISH) for both targeting genes and nuclei visualization. For multi-layer scanning at  $N=6$ , 13, and 26 layers at 1, 0.4, and 0.2  $\mu\text{m}$  interval respectively were performed on the same regions of interest which were identified beforehand. Scanning was performed in semi-automated mode. The exposure time of the scans were set based on the signal intensities of each channel. The scanned images were JPEG compressed, i.e. JPEG compression ratio was set to 80, and images were saved on the local drive of the computer used to drive the scanning. Scanning time and file size of each scan were recorded. Z-stack and extended focus were performed on the regions for multi-layer scanning.



## 2.4 Image evaluation and analysis

The quality of the scanned images by wide-field and confocal was assessed visually with the viewing software CaseViewer 2.1 provided by 3DHISTECH in parallel for comparison. The 3D reconstruction, quantification of dots, and co-localization analysis were conducted with Imaris 8.1.2 (Bitplane, Zurich, Switzerland). Briefly, WSI images were annotated in CaseViewer based on the morphology of nuclei and the fluorescence signals inside, and the selected tumor regions were exported into TIFFs with individual channels separated. The exported TIFFs were then imported into Imaris for quantification, co-localization, and 3D reconstruction analysis. The surface detection function in Imaris was initially used to segment the DAPI stained nuclei. A mask channel created from the segmentation result was then utilized to limit the analysis in other channels within the nuclei. The dots located inside the nuclei were finally quantified through the spots detection function. Co-localization analysis of dots were performed for both Z-stack and extended focus scanned ALK FISH images using the Imaris extension tool. The cut-off distance between FITC and TRITC to define fused (co-localized) signals was set as 1.2  $\mu\text{m}$  for the ALK break-apart FISH probe. The statistic data of quantification and co-localization were exported out of Imaris and analyzed further in Excel (Microsoft, Washington, USA). The two-tailed Student t-Test and ANOVA were used to compare data, and a  $p < 0.05$  was considered indicative of statistical significant difference.

## 3. Results

### 3.1 Confocal scanning provides sharper images than wide-field scanning

While the current scanner provides both confocal imaging and wide-field imaging, confocal scanned images were observed to be sharper with higher contrast and less noise than wide-field scanned images. The subcellular details including nuclear contours and fluorescent dots were therefore visualized more clearly in confocal images than those in wide-field images especially when viewed at high magnification (Figure 1A). The ability of doing optical section by confocal imaging allowed the Z-stack images to be reconstructed for 3D rendering, while wide-field Z-stack images looked blurry for structure and contour of both the nuclear and fluorescent dots after 3D reconstruction (Figure 1B). The 3D reconstruction generated from Z-stack of confocal scanning was dramatically improved in comparison with that from wide-field scanning. Thus confocal images of FISH slides afforded more reliable information for both diagnosis and



research than wide-field images. Compared to wide-field scanning, however, confocal scanning required a little more time with the same settings (exposure time of each fluorescent channel, the size of scanning area, the number of layers, and the focus method), while took up much larger storage (Figure 1C). Nevertheless, the higher image quality weights more than the longer scanning time and larger storage as accurate FISH analysis depends on the image with high quality. Confocal scanning therefore was chosen for FISH slides imaging in the following study.

### **3.2 Multi-layer Z-stack scanning increases dots detection as well as the scanning time and file size than single-layer scanning**

Fluorescence dots labeled target genes are extremely tiny, and do not distribute in the same level in nucleus, which requires high-resolution imaging system as well as the multi-layer imaging strategy. The confocal imaging technology provides high spatial resolution for visualization and quantification analysis of the dots from various channels with distinguishable colors. As show in Figure 2 A&B, the single-layer image did not present the dots above and/or below the focal plane, which results less number of dots by the quantification image analysis from single-layer image compared to multi-layer. Multi-layer Z-stack images had more number of fluorescence dots in both FITC and TRITC channels than single-layer image at the same imaging area ( $p < 0.01$  in ALK FISH and  $p < 0.05$  in EGFR FISH compared the single-layer image with 6-layer, 13-layer, and 26-layer images). Although the numbers of dots in both FITC and TRITC of 6-layer images were less than those of 13-layer or 26-layer images, there was no statistically significant difference between 6-layer with 13-layer or 26-layer ( $p > 0.05$ ). There was no difference of numbers of dots in both signal channels between 13-layer and 26-layer from the quantification analysis. The multi-layer Z-stack scanning improved the accuracy of dots detection for quantification analysis and nuclear scoring based diagnosis; however, it increased the scanning time and image file size as well (Table 1), both of which were proportional to the number of scanned layers.

### **3.3 Z-stack provides more image information than extended focus**

For multi-layer scanning, both extended focus and Z-stack can image all the dots in different focal planes by collecting the fluorescence signal through Z-axis with a small interval between each focal plane (0.2 to 1  $\mu\text{m}$ ). The quantification of dots in nuclei showed that both extended focus and Z-stack yielded more dots in nuclei than single-layer images (data not shown).



Extended focus, however, saved highest signal intensity through the Z-axis, and ignored the spatial and volume information of dots through Z-axis. In the contrast, Z-stack saved both fluorescence signal intensity and the location of each dots, providing more image information than extended focus. Thus the image file size of multi-layer extended focus was approximately equal to single-layer, while the scanning duration was close to same layers of Z-stack (Figure 1C). The number of dots inside nuclei analyzed based on extended focus images was smaller compared with Z-stack at the same scanning area (Figure 3). These indicate that the algorithm of extended focus might reduce the actual number of intra-nuclear dots especially when overlapping dots which are close to each other in X-Y plane but separated in Z-axis frequently appear in image. This might result in inaccurate interpretation of genetic information of mutation. Z-stack keeps the distance information between dots in Z-axis, which can be used to quantify the number of dots more accurately.

### 3.4 Extended focus increases the chance of co-localization

Various FISH assays, e.g. ALK FISH using break-apart probe, require the co-localization analysis to judge separation or not of dots from different channels for the diagnosis of gene translocation and/or fusion. The spatial distance between different colored dots is essential for the correct analysis of probe splitting and diagnosis of mutation. As shown in Figure 4A&B, compared to multi-layer Z-stack, the automated co-localization analysis of dots from FITC and TRITC channels explained that extended focus increased the chance of co-localization. This is considered to be due to the ignorance of distance in Z-axis in extended focus algorithm, while the distance in X-Y plane between dots is not significant but the actual three-dimensional distance is significant. The false co-localization may result in incorrect interpretation of nuclei for gene translocation by incorrect analysis of splitting probes (Up-right nucleus in Figure 4B). Z-stack does not ignore the distance in Z-axis between dots from different channels, which information combined with the distance in X-Y plane can be used to measure the three-dimensional distance between dots accurately. However, there was no significant difference of the overall ratio of co-localization between Z-stack image and extended focus image (Figure 4C). Further study is needed to clarify whether extended focus affects the scoring of break-apart and/or fusion FISH assays for cytogenetical diagnosis.





### **3.5 Computer-assisted diagnosis by automated quantification of signals in each nucleus**

For most FISH tests, the diagnosis of positive cells and the cytogenetic diagnosis of sample rely on the number and/or local relationship of dots inside each individual nucleus. In some special FISH tests, two or more fluorescence probes with distinguished colors are applied to one sample (Multi-gene FISH), which are able to visualize a variety of target genes simultaneously (Figure 5A) [16, 17]. The current workflow to interpret FISH tests by manually counting dots and scoring nuclei under fluorescence microscope takes a lot of time and efforts especially for those of multi-gene FISH or those with multiple copies. The studied confocal scanner is capable to image up to 9 fluorescence channels and generate the composite digital image assigning each channel with a unique pseudo-color. Both quantification and co-localization analysis can be done at the single cell level by computer-assisted analysis of these confocal scanned FISH images. The statistical data generated by digital image analysis on a single cell level can be used to assist cytogenetic diagnosis of amplification, deletion, and translocation. The Table 2 is an example for image analysis based diagnosis support from an ALK FISH assay.

### **3.6 Three-dimensional reconstruction analysis of FISH for molecular research**

Often times, it's very meaningful in researches to investigate the spatial organization and relationship of the nucleus, chromosome, and gene as well as their functions by FISH [18, 19]. However, the individual genes visualized by fluorescence probes are extremely tiny and distribute in various levels inside nucleus. A two-dimension imaging strategy like one-layer imaging or extended focused imaging is not capable to provide spatial information of nucleus and gene. Confocal imaging has the capacity to conduct optical sectioning through multi-layer Z-stack scanning, which allows 3D reconstruction of nucleus and provides the possibility for further deep three-dimensional analysis. As shown in Figure 5, a three-dimensional reconstruction of FISH Z-stack images with 4 distinct channels was presented. The number of dots in each channel was quantified automatically (Figure 5A), and the line distance and angle between dot to dot were measured in three-dimensional space to support structural and functional research in molecular level (Figure 5B).



#### 4. Discussion

In this study, we have shown that FISH slides can be digitized using a confocal fluorescence scanner with high-resolution images in both single-layer and multi-layer (Z-stack and/or extended focus) modes. Confocal scanning provided images with higher quality in terms of sharpness, contrast, and noise when compared to wide-field fluorescence scanning. While requiring longer scanning time and larger image file storage, multi-layer Z-stack scanning provided more image information than single-layer; extended focus decreased the storage down to the size close to single-layer scanning, but induced imprecise image analysis. Automated quantification and co-localization analysis at the single cell level provided computer-assisted cytogenetic diagnosis of FISH assay, and 3D reconstruction allowed deep investigation of the spatial organization of nucleus and gene for research.

Hybridized dots, which carry the specific genetic information on FISH slides, have extremely small size and occupy tiny volumes inside the nuclei. Determined by the spatially modulated illumination microscopy, three gene domains (c-myc, p53, and p58) in human lymphocyte cell nuclei were reported to have 103 nm, 119 nm, and 123nm in average diameter [20]. The fluorescent dots of ALK and EGFR FISH in the current study were measured to have an average diameter of 500 to 1000 nm. To visualize and distinguish these tiny dots, it requires microscopy with high magnification objective. In cytogenetic laboratory, the epifluorescence microscopy (wide-field fluorescence microscopy), which has an oil objective with magnification more than 60× and NA larger than 1.0, is commonly used to view FISH slides and count the fluorescent dots for scoring and diagnosis. Most WSI scanners in market have 20× or 40× objectives for digital fluorescence imaging, and they digitize slide with optics similar to epifluorescence microscopy [9, 21]. These scanners have difficulty to capture the signals from the miniscule fluorescence dots in nucleus when digitizing FISH slide. Confocal imaging technology increases optical resolution compared to traditional wide-field fluorescent imaging by means of adding a spatial pinhole placed at the focal plane of the lens to eliminate the out-of-focus light [22]. The confocal scanner used in current study was implemented with a high magnification of 40× water immersion objective (NA 1.2), producing final image with high pixel resolution of 0.1625 μm/pixel, which is capable to acquire the signal from each fluorescence dot from FISH slide.



Besides, as shown in Figure 2, the tiny fluorescent dots could not be completely detected by a single-layer scanning method due to their three-dimensional spatial distribution. In cytogenetic laboratory, microscopy objective needs to be tune up and down in order to screen and count all the tiny fluorescence dots located in various levels inside nucleus. As for the digitized imaging, multi-layer scanning including both Z-stack and extended focus, a simulation process of moving microscopy objective up and down manually, provides complete signal information for quantification based scoring and diagnosis compared single-layer scanning. Interestingly, the difference of numbers of dots between single-layer and multi-layer in ALK FISH is more significant than that in EGFR FISH (Figure 2A&B). When carefully looking at the distribution of dots in Z-stack images, dots in ALK FISH were found to distribute frequently in various layers while in EGFR FISH they are relatively localized in same layer. In addition, there was no significant difference of the number of dots among 6-layer, 13-layer, and 26-layer, which indicates that for dots with diameter range of 500-1000 nm, the 6-layer scanning with 1  $\mu$ m interval is capable to capture the signals from all dots. Taken the longer scanning time and larger file size, 13-layer and 26-layer may not be needed to enumerate dots with size close to 500-1000 nm for scoring and diagnosis. However dots with smaller size in certain FISH assays might need more than 6-layer scanning to capture all signals. Based on the above discussion, multi-layer scanning has to be taken for FISH imaging rather than single-layer to avoid the incapability of capturing all signals, and the number of layers will depend on the size and distribution of dots.

Multi-layer Z-stack produced more dots information than single-layer; however, it increased the scanning time and image file size tremendously as more layers were scanned. The algorithm of extended focus adopted by many slide-scanning machines saves the storage but allows preservation of critical image information [12, 21]. As for the 3DHistech Panoramic Confocal scanner used in the current study, after choosing the option of extended focus before initiating scanning, the built-in algorithm (maximum intensity projection in this study) will select the sharpest image from every focal level for each image field, and combine them into one single image. Therefore those parts that were blurry (out of focus) will also become sharp in the final image. This method guarantees the maximum depth of sharpness but reduces digitization speed and increases scanning duration. Similar to Z-stack, the digitizing speed of extended focus was also proportional to the number of scanned layers, while extended focus took up as little storage as the single-layer scanning (Figure 1C). Compared with Z-stack, extended focus could save much



storage, and accelerate the speed of the image load and transfer for data viewing, sharing, and analyzing. Both multi-layer Z-stack and extended focus scanned images provide more image information than single-layer images, because both of them collect the information from multiple focal planes through the whole depth of sample. Extended focus, however, might result in reduced number of intra-nuclear fluorescent dots (Figure 3A-D) and increased frequency of co-localization than Z-stack (Figure 4A&B). The ignorance of the spatial distance in Z-axis of extended focus is the main reason responsible for these differences compared with Z-stack. Although we demonstrated there's no difference of statistical significance between multi-layer Z-stack and extended focus in quantification and co-localization analyses (Figure 3E and Figure 4C), further study is needed to validate whether extended focus has inconsistent scoring and cytogenetic diagnosis with Z-stack for both amplification and break-apart FISH probes. Additionally, future studies could compare the scoring results of automated image analysis with the manual scoring.

It is meaningful to investigate the spatial organization and alteration of the chromosomes and genes by FISH for cancer research. The organization chromosomes may reflect their functions such as replication, transcription, repair, and recombination processes. And the relative position of genes could contribute to chromosomal rearrangements [18, 19]. As mentioned above, the individual genes visualized by fluorescence probes localize in various levels inside nucleus, which can not be interpreted accurately by a two-dimension imaging strategy such as a single-layer or extended focus imaging. With an epifluorescence microscopy, people can tune the objective up and down in order to visualize the spatially distributed fluorescence dots, but it's impossible to measure the distance between hundreds of dots by eye precisely. Confocal Z-stack imaging provides the possibility to do optical sectioning of FISH slide and reconstruct the serial sections into 3-dimensional volume, which permits to localize the interesting genes precisely in nucleus and detect the distance between genes (Figure 5B) and measure their distance from nuclear border or center precisely. This provides a useful tool for cancer molecular research by imaging and 3-dimensional reconstruction analysis.

## 5. Conclusion

Confocal provides sharper images than wide-field for FISH slide scanning. Multi-layer not single-layer scanning is necessary for FISH imaging. Extended focus reduces file size and storage, but it



could result in inaccurate analysis compared with Z-stack. Z-stack scanning provides high volume image information for spatial analysis. We foresee confocal multi-layer scanning as a digital pathology application tool for FISH imaging in both clinical diagnosis and cancer research in future.

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### Conflict of Interest

Authors declare no conflict of interest.

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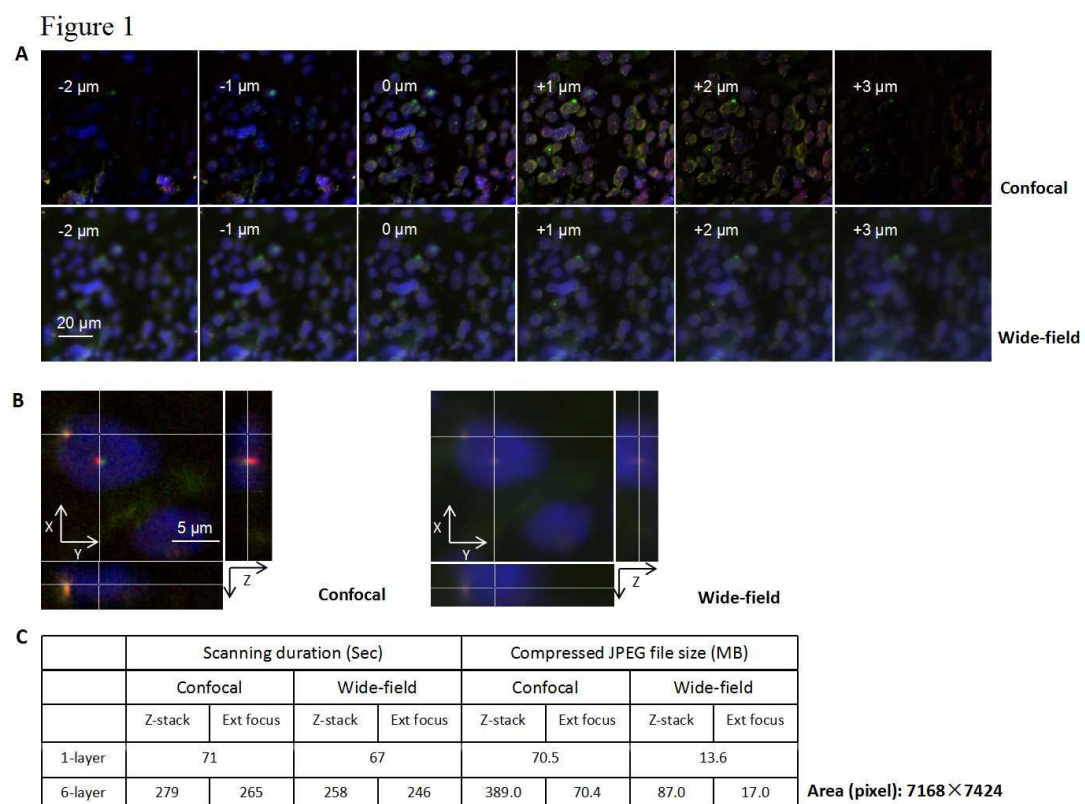


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## FIGURE LEGENDS

**Figure 1** The comparison of wide-field and confocal scanning. (A) Image quality comparison of serial images through the Z-axis of 6-layer Z-stacks scanned by confocal and wide-field. (B) 3D reconstruction of the 26-layer Z-stacks scanned by confocal and wide-field. (C) Scanning speed and image file size comparison of confocal with wide-field, single-layer with multi-layer, and Z-stack with extended focus. Slide was ALK FISH. Blue is nucleus, green is FITC channel, and red is TRITC channel.

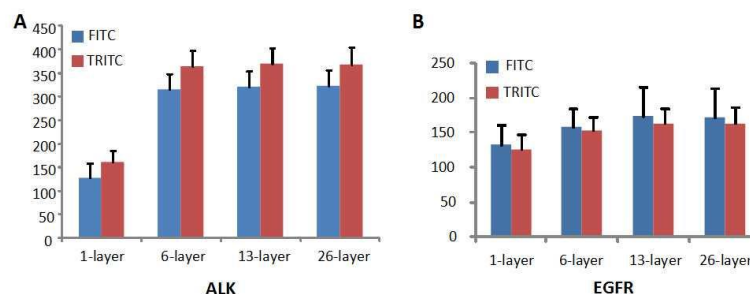






**Figure 2** The comparison of intranuclear dots between single-layer and multi-layer Z-stacks. (A) The average number of dots in both FITC and TRITC channels in ALK FISH image ( $110\ \mu\text{m} \times 110\ \mu\text{m}$ ) detected in Imaris automatically. (B) The average number of dots in both FITC and TRITC channels in EGFR FISH image ( $110\ \mu\text{m} \times 110\ \mu\text{m}$ ) detected in Imaris automatically. Data are expressed as mean  $\pm$  SD from eight sets of images.

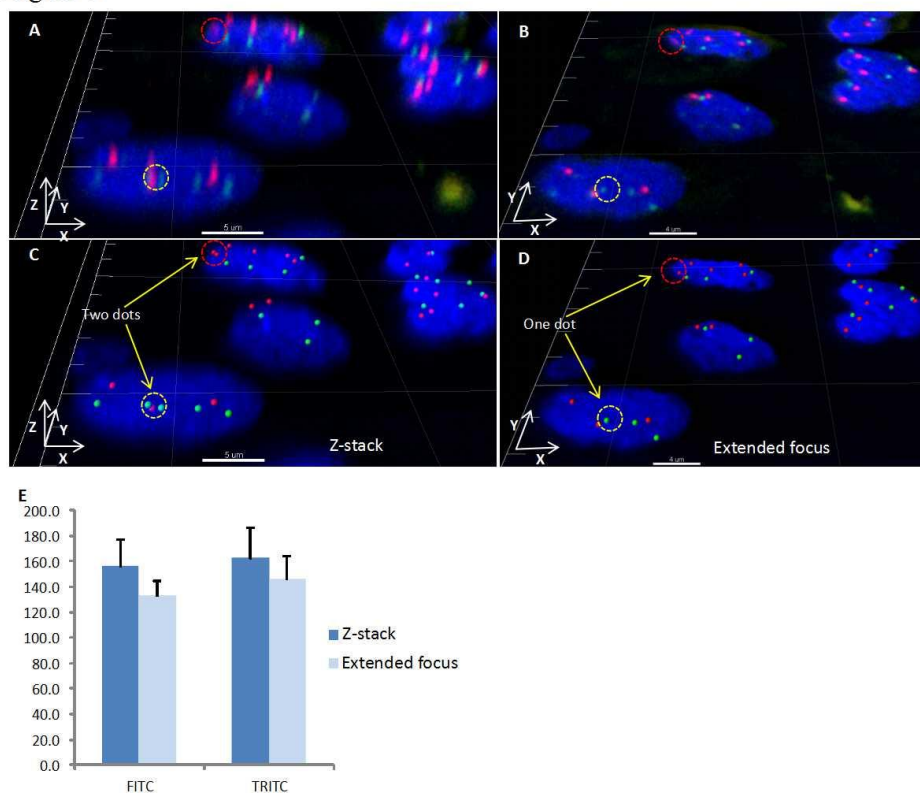
Figure 2





**Figure 3** Comparison of extended focus and Z-stack for quantification analysis of dots inside nucleus. (A&B) Extended focus and Z-stack images scanned with 26-layer and 0.2  $\mu\text{m}$  interval at the same area; (C&D) Dots detected automatically from images in (A&B) in both FITC (green) and TRITC (red) channels by Imaris. (E) The comparison of average numbers of dots in EGFR FISH images ( $110 \mu\text{m} \times 110 \mu\text{m}$ ) scanned by extended focus and Z-stack. Blue is nucleus, green is FITC channel, and red is TRITC channel.

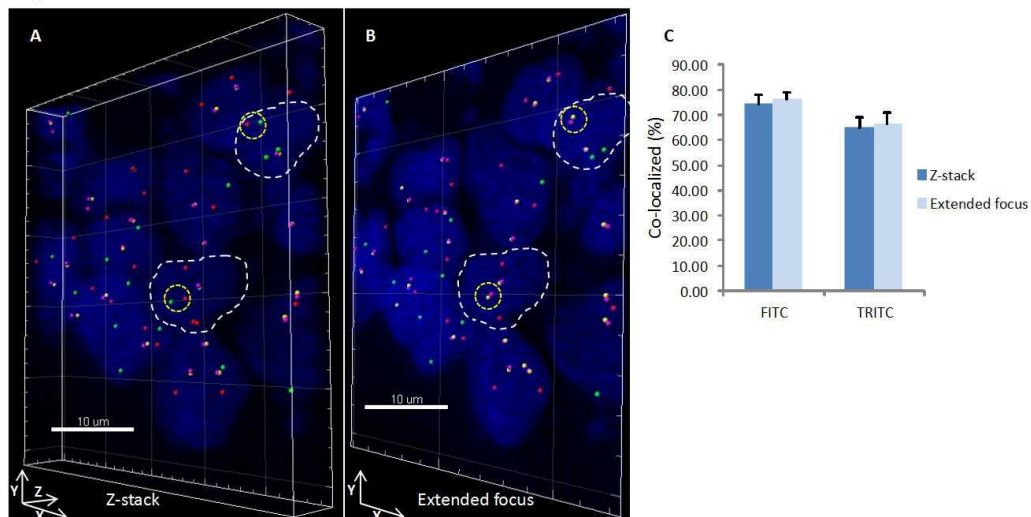
Figure 3





**Figure 4** Comparison of extended focus and Z-stack for co-localization analysis of FITC and TRITC channels to detect break-apart. (A) The co-localization analysis in a 26-layer Z-stack of ALK FISH. (B) The co-localization in a 26-layer extended focus image of ALK FISH at the same region of (A). Yellow and purple dots are co-localized FITC and TRITC dots. Green and red dots are break-apart FITC and TRITC dots. Dots with distance less than  $1.2\ \mu\text{m}$  were defined as co-localized. Blue is nucleus. (C) The dots quantification and ratio of co-localization from 26-layer of Z-stack and extended focus. Data are expressed as mean  $\pm$  SD from eight sets of images ( $50\ \mu\text{m} \times 50\ \mu\text{m}$ ).

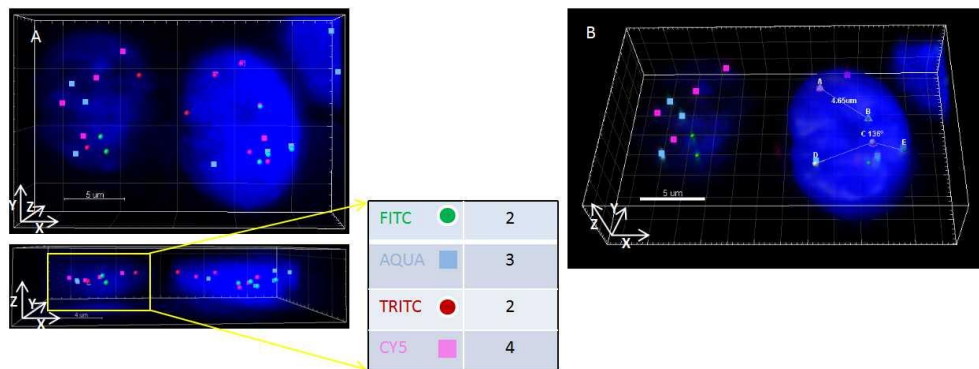
Figure 4





**Figure 5** 3D reconstruction and measurements in 3-dimensional volume. (A) 3D volume rendering from Z-stack (26-layer, 0.2  $\mu\text{m}$  interval) of multi-gene FISH was reconstructed in Imaris and the dots in each individual channels were detected and quantified automatically in single cell level. (B) Measurements of distance and angle between fluorescent dots 3-dimensionally. Blue is DAPI for nuclear counterstaining.

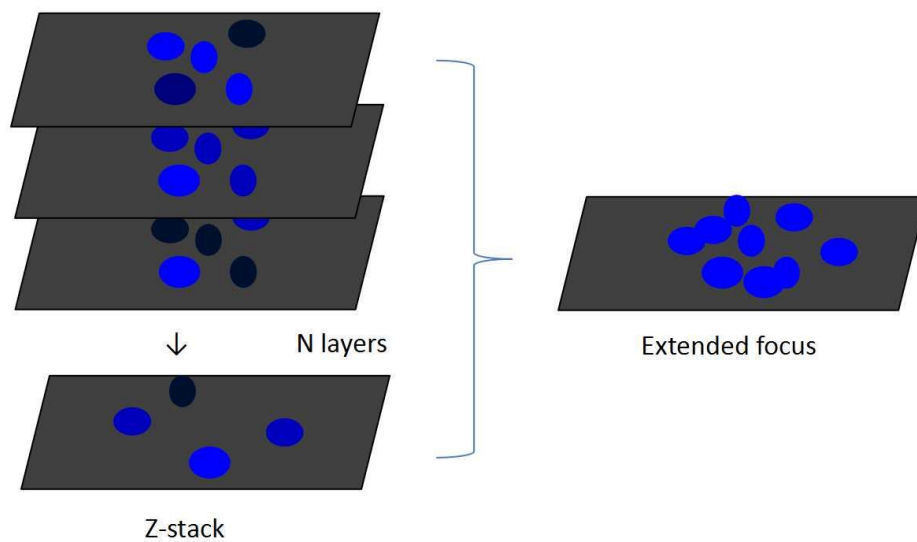
Figure 5





**Supplementary Figure 1** The schematic illustration of Z-stack imaging (left) and extended focus imaging (right). Light blue indicates in focus or high signal intensity while dark blue indicates out-of focus or low signal intensity.

Supplementary Figure 1





**Table 1** Comparison of scanning duration and file size by confocal scanning

	Area size	File size	Scanning time
1-layer	4864 x 4608 pixel <sup>2</sup>	25.94MB	27s
6-layer Z-stack	4864 x 4608 pixel <sup>2</sup>	141.71MB	1m 54s
13-layer Z-stack	4864 x 4608 pixel <sup>2</sup>	278.87MB	4m 02s
26-layer Z-stack	4864 x 4608 pixel <sup>2</sup>	542.03MB	7m 44s



**Table 2** Quantification and co-localization analysis of dots in each nucleus for scoring and diagnosis

Nuclei	FITC		TRITC		Classification
	Co-localized	Non-co-localized	Co-localized	Non-co-localized	
1	2.00	1.00	2.00	2.00	+
2	1.00	1.00	1.00	1.00	+
3	2.00	1.00	2.00	2.00	-
4	2.00	1.00	2.00	2.00	+
5	3.00	1.00	3.00	1.00	+
6	2.00	1.00	2.00	2.00	+
7	2.00	2.00	2.00	1.00	+
8	2.00	1.00	1.00	3.00	+
9	2.00	1.00	2.00	1.00	+
10	3.00	0.00	3.00	0.00	-
11	3.00	0.00	3.00	0.00	-
12	3.00	0.00	3.00	0.00	-
13	2.00	1.00	3.00	1.00	+
14	3.00	0.00	3.00	0.00	-
15	3.00	0.00	3.00	0.00	-
16	6.00	0.00	6.00	0.00	-
17	2.00	0.00	2.00	0.00	-
18	4.00	0.00	4.00	0.00	-
19	2.00	0.00	2.00	0.00	-
20	2.00	1.00	2.00	2.00	+