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Two-Photon Multicolor FISH: A Versatile Technique to Detect Specific Sequences within Single DNA Molecules in Cells and Tissues

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Abstract

Two-photon multicolor fluorescence *in situ* hybridisation (FISH) is presented as an advanced single molecule detection technique. Based on a two-photon excitation process it enables pinhole-free 3D laser scanning microscopy. Nonresonant two-photon absorption in a sub-femtoliter excitation volume was realized with a femtosecond laser scanning microscope equipped with a high numerical aperture objective. Nonlinear near infrared laser excitation and multicolor FISH has been used to image a variety of specific DNA regions. This innovative single molecule technique was applied to visualize genomic regions in fiber-DNA, human metaphase chromosomes, interphase nuclei and histological sections. Intense 170 fs laser pulses at 800 nm and GW/cm^2 intensities have been employed to induce visible fluorescence of a variety of FISH fluorophores coupled to DNA probes. In particular, two-photon excited FITC-labeled 40 kb probes and multicolor labeled centromeric probes that bind to repetitive sequences of 0.340-2.000 kb have been used to visualize subtelomeric and different centromeric regions in metaphase chromosome spreads. Spectrum Orange and Spectrum Green labeled bcr and abl gene probes that target a stretch of 300 kb and 650 kb, respectively, have been imaged in a single fiber-DNA molecule with an estimated number of fluorescent molecules of 75 - 225 μm^{-1}.

Using the advantages of pinhole-free optical sectioning with submicron spatial resolution and multi-fluorophore single-wavelength-excitation, 3D images of multiple labeled centromeric regions of amniotic fluid cells in interphase have been obtained and used in diagnosis of trisomy 18. In addition, first 3D two-photon studies on centromer distribution in human kidney biopsies by labeling chromosomes X, Y and 4 with the FISH-fluorophores Spectrum Blue, Spectrum Green and Spectrum Orange have been performed. As demonstrated, two-photon Multicolor FISH has the potential to perform Multi-Gene-Imaging with high spatial resolution also in turbid tissue layers.

Introduction

Progress in molecular cytogenetics, such as the development of FISH combined with DNA probe technology, and advances in microscopy such as the introduction of laser scanning microscopy and digital fluorescence microscopy has revolutionized the identification and imaging of certain genomic regions of the biomolecule DNA. Driven by the Human Genome Project and its first complete preparation of the genomic map of chromosome 22 in 1999 [1], a large variety of well defined nucleic acid sequences as recombinant clones and chemically synthesized oligonucleotides have been developed for gene detection.

A normal cell (except germ cells) contains exactly 2 (before DNA duplication) or 4 (after DNA duplication) sets of DNA macromolecules. The DNA macromolecules in the human genome contain $3 \cdot 10^9$ base pairs (3 Gbp) with an average gene concentration of 22 - 27 genes per megabase (Mb). The mean size of the 65,000 to 80,000 genes is 10–15 kb with a mean distance between genes of 25-33 kb. A chromosome has an average of 3,000 genes. The smallest chromosome 21 contains 46 Mbp. According to the Watson-Crick model of the double-stranded DNA helix, the distance of 3.4 Å between consecutive base pairs results in a 1.5 cm long and 2 nm thick DNA macromolecule in this chromosome [2, 3]. The metaphase chromosome of about 2 µm is therefore condensed by a factor of about 7,000 due to complex DNA packaging. With a typical lateral resolution of about 0.3 µm in standard light microscopes, about 7 Mb can be resolved in this case of highly condensed chromatin. However, when using fiber-DNA under optimum conditions, where the DNA molecule is decondensed to the level of the double helix and released from the nucleus in long thin fibers, a resolution of 1 kb corresponding to 0.34 µm in length can be achieved.

FISH, based on hybridization of specific DNA probes to single stranded DNA or RNA molecules and their detection by one-photon excited fluorescence, has become an important cytogenetic technique for gene mapping, clinical cytogenetics (prenatal/postnatal and tumor diagnostics), germ cell analysis and comparative genomic hybridization (CGH). Normally, the length and concentration of DNA probes is between 0.25 kb and 1.00 kb and 1.5 and 50 ng/ml, respectively [4]. A typical value for the number of fluorescent molecules directly bound to a DNA probe (incorporation) is 1-3 per 100 bases which would correspond to 2 to 30 molecules in a typical fluorophore-labeled probe [5].

One of the important features of FISH is the possibility to detect multiple fluorescent targets with different emission wavelengths [6-11]. Multicolor-FISH (M-FISH, also known as multiplex-FISH) provides therefore the possibility of simultaneous Multi-Gene-Imaging. Unfortunately, different excitation wavelengths in the ultraviolet (UV), blue and green range are typically required in conventional M-FISH to induce the visible fluorescence of the most common FISH fluorophores and of the unspecific DNA stain DAPI (4',6-diamidino-2-phenylindole, counterstain). Multi-excitation wavelengths are generally derived e.g. by special multicolor filters, lasers with multi-line output [12] or filter wheels.

Conventional FISH technique is based on one-photon microscopy with UV and visible fluorescence excitation radiation at low intensity. Typically, high-pressure mercury or xenon lamps are used as excitation sources. Also, FISH with confocal laser scanning microscopes, based on the application of argon ion lasers and helium neon lasers with radiation in the blue/green and red spectral range, has been reported e.g. [13-16]. These microscopes provide also the capability of optical sectioning and 3D imaging of FISH fluorophores. Unfortunately, the most common DNA counterstain DAPI as well as blue-emitting FISH fluorophores cannot be laser-excited with such equipment. A disadvantage is also the large excitation volume leading to fluorophore photobleaching and sample photodamage in out-of-focus regions. This limits the potential of conventional 3D microscopy to study genomic regions in interphase nuclei, embryos and tissue sections. Furthermore, disturbing background fluorescence from non-target material in out-of-focus regions may be induced. A further disadvantage is the low fluorescence detection efficiency due to the transmission of the fluorescence photons through scanning optics and through the pinhole in front of the detector.

In this paper we report a novel single molecule imaging technique based on the combination of two-photon fluorophore excitation using ultrashort laser pulses in the near infrared (NIR), three dimensional laser scanning microscopy and M-FISH technology. This technique, named two-photon multicolor FISH, is used for imaging of specific regions of the human DNA macromolecule in fiber-DNA, chromosomes, interphase nuclei and tissue sections. As demonstrated this technique allows excitation of a variety of conventional FISH-fluorophores and DNA counterstains within a sub-femtoliter excitation volume with a single NIR wavelength.

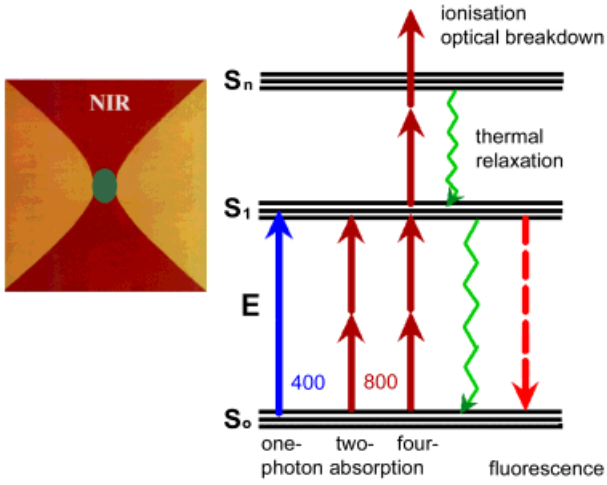


Fig. 1. *Principle of highly localized two-photon fluorophore excitation with intense near infrared laser radiation. In contrast to conventional fluorescence excitation with high-energy photons (e.g. 400 nm), non-resonant two-photon induced fluorescence is based on the simultaneous absorption of two low-energy photons (e.g. 800 nm) at MW/cm^2 and GW/cm^2 intensities. A highly localized sub-femtoliter fluorescence excitation volume (green-colored region in the left image) can be realized by diffraction-limited focusing with NA>1 objectives. At TW/cm^2 intensities, multi-photon processes with at least four photons induce destructive optical breakdown and plasma formation in the biological target.*

Materials and Methods

Principle of Two-Photon Fluorophore Excitation and Two-Photon Laser Scanning Microscopy

Two-photon excitation of electronic states, based on the simultaneous absorption of photons, was predicted in 1931 [17] and first realized with the availability of lasers 30 years later [18]. The first two-photon excited fluorescence imaging of biological specimens with NIR radiation was published in 1990 [19].

Two-photon fluorophore excitation in Life Sciences is based on the application of NIR radiation in the spectral range of 700 - 1200 nm. Water with the low absorption coefficient of ≤ 0.1 cm^{-1} [20] is considered to be the major one-photon absorber in DNA targets in this spectral range which is also referred as "optical window" of cells and tissues. Two-photon excitation is based on high photon concentration in space and time. Due to the relatively low two-photon and three-photon absorption cross sections of fluorophores of about 10^{-48} to 10^{-50} cm^4 s/photon and 10^{-75} to 10^{-84} cm^6 $(s/photon)^2$ [21,22], respectively, non-resonant two-photon excitation requires photon flux densities of $>10^{24}$ photons $cm^{-2}s^{-1}$. Using radiation in the spectral range of 700 - 1100 nm, NIR intensities in the range of MW/cm^2 to GW/cm^2 are necessary which can be provided by diffraction limited focusing continuous wave (cw) laser beams with high numerical aperture (NA) objectives [23]. Using NA>1 objectives, the area of high intensity and fluorescence excitation is confined to a sub-femtoliter focal volume via the simultaneous absorption of two or three photons with approximately half or one third of the energy required for one-photon fluorophore excitation (Fig. 1). Considering a fluorescence excitation volume of 0.1 fl [19] and a 10 nM fluorophore solution, only one molecule would be located within this tiny volume.

Despite the enormous intensities, the cellular heating rate is below 2K/100 mW in most biological targets due to the negligible one-photon absorption [24, 25]. Two-photon fluorescence imaging using cw lasers (Ti:sapphire laser, krypton ion laser) in combination with a scanning unit has been reported [26, 27]. The efficiency of two-photon excitation follows a power squared relation. However, trapping effects and photothermal effects limit the use of high power cw sources for fast fluorescence scanning microscopy with µs beam dwell times per voxel. Much more efficient in fast two-photon fluorescence imaging is the use of high repetition pulsed laser systems with moderate peak power in the W/kW range but with mW mean power. The efficiency of two-photon excitation and the fluorescence yield follows the following relation [19]

$$n \approx P^2\alpha/(\tau f^2)\pi^2 NA^4/(h^2c^2\lambda^2)$$

with h: Planck's constant, c: light speed, n: number of absorbed photon pairs, P: mean power, α: molecular two-photon absorption coefficient, τ: pulse width, f: repetition frequency, NA: numerical aperture, λ: wavelength.

Today's two-photon microscopes are typically based on the application of mode-locked Ti:sapphire lasers at 76-80 MHz repetition frequency. Because the fluorescence yield depends on a P^2/τ relation in two-photon microscopy, the efficiency increases for high peak power and low pulse width. It means also, that microscopy with laser pulses of 1 ps requires only a factor of 3 higher mean power compared to 110 fs pulses in order to achieve same fluorescence images [28]. In order to perform fast imaging, the microscopes are equipped with beam scanners.

The spatial resolution in two-photon fluorescence microscopy is similar to one-photon confocal laser scanning microscopy with typical values of $\lambda/2$ full width half maximum (FWHM) in lateral dimension and λ FWHM in axial direction. The axial resolution can be enhanced by about 50 % by the additional use of a confocal pinhole [29]. Schrader and Hell have demonstrated axial resolutions of about 145 nm by 4π two-photon microscopy [30]. The theoretical resolution of three-photon microscopy at 700 nm has been reported to be < 200 nm in the radial direction and about 500 nm in the axial direction [22, 31].

Although the exact two-photon and three-photon molecular cross sections are not known for most fluorophores in cell biology and biotechnology, it has been demonstrated that non-linear excitation of a fluorophore with the main one-photon absorption peak at λ and 40 nm FWHM would require a NIR excitation wavelength of $2\lambda\pm40$ nm for two-photon excitation and $3\lambda\pm60$ nm for three-photon excitation [22]. Nonlinear excitation of a variety of conventional fluorophores was demonstrated including two-photon and three-photon excitation of the fluorescent DNA stains DAPI and Hoechst 33342 which bind preferentially to Adenine-Thymine rich regions in the minor grove of the DNA [32].

Two-photon microscopy can be performed without damage to the biological target within a certain intensity-dependent optical window. For example, femtosecond laser microscopy on living specimens has been performed with a peak intensity of about 200 GW/cm^2 for hours without impact on cellular reproduction and vitality [28]. Squirrell et al. [33] exposed hamster embryos for 24 hours with NIR femtosecond laser pulses of a two-photon fluorescence microscope without impact on birth in contrast to conventional one-photon laser scanning microscopy. However, above certain intensity thresholds a two-photon photodestruction process occurs. For same pulse energy, this process was found to be more pronounced for shorter pulses. If increasing the intensity in the range of TW/cm^2, a further destructive process may occur based on intracellular optical breakdown and plasma formation [28].

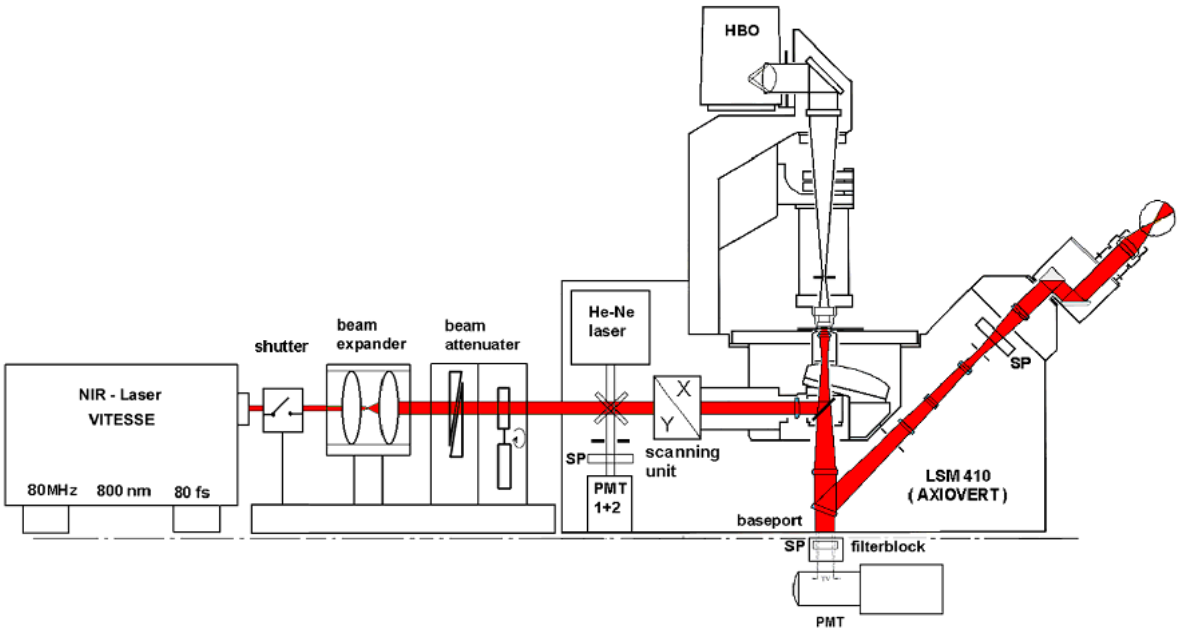


Fig. 2. *Experimental setup for two-photon multicolor FISH with near infrared femtosecond laser pulses.*

Laser Microscope

The 800 nm beam of a mode-locked 80 MHz Ti:sapphire laser (Vitesse, Coherent, Santa Clara, USA with 1 W output power and 80 fs output pulse width) was expanded by an 1:4 Galileian telescope and coupled to a modified inverted laser scanning microscope (LSM 410, Carl Zeiss Jena GmbH, Jena, Germany) with x,y galvanoscanners and focused to a diffraction-limited submicron spot size with a phase contrast Plan-Neofluar objective (63x, NA=1.25) or a 40x oil immersion objective (NA=1.3) (Fig. 2). The short pulse width implies an excitation wavelength range of 800±6 nm. The transmission through the optical system results in pulse broadening due to group velocity dispersion. The pulse duration at the sample was determined to be 170 fs using an autocorrelator (APE, Berlin, Germany). The mean laser power was measured in air after transmission through the objective with the power meter Fieldmaster FM (Coherent, Santa Clara, USA) and adjusted to be typically 5 - 7 mW for nonlinear fluorescence excitation and <1 mW for transmission imaging. At 5 mW mean power, the peak power and the peak intensities reaches values of 0.4 kW and 0.6×10^{12} W/cm^2, respectively, when assuming a full width half maximum beam size of $\lambda/2NA \approx 310$ nm [28]. A typical beam dwell time per pixel of ≈4 µs per scan was chosen (1 s/frame, typical zoom 4, 512 x 512 pixels covering a 80 µm x 80 µm sample area, pixel dimension 0.16 µm). With a typical chromosome area of 6 to 20 µm^2, the beam dwell time on the chromosome was 1-3 ms per scan. Sometimes the frame rate was reduced to 0.125 s^{-1}. For nonlinear 2D imaging normally one scan was performed whereas 3D imaging was performed in z steps of 0.5 µm or 1 µm. Multi-photon excited fluorescence was measured with the photomultiplier R1527P (Hamamatsu Photonics, Japan) mounted to the modified baseport of the microscope. This allows highly efficient collection of fluorescence photons without fluorescence transmission through the scanning optics and pinhole units. A 700 nm short pass filter prevented the scattered laser radiation from reaching the detector. In addition, a variety of long-pass filters and bandpass filters have been used to differentiate between different emitting fluorophores. An additional low-sensitivity detector was used for transmission microscopy at 800 nm. The fluorescence images were pseudo-color coded.

DNA Material and Chemicals

Human metaphase chromosomes from peripheral blood were prepared by standard methods [34,35]. Uncultured amniocytes were directly prepared from samples taken from pregnant woman in the 16 – 20 weeks of gestation. Cell preparation ends up with a cell suspension fixed in methanol / 100% acetic acid (3:1) which was carefully applied to a slide.

The fiber DNA – probes, prepared from cells which have been first embedded in low melting agarose, digested with proteinase K and then stretched after melting of the agarose [36] were kindly provided by Ritva Karhu and Minna

Tanner (Center for Laboratory Medicine, Tampere University Hospital, Finland).

The centromer specific α- satellite probe (except chromosome 1 which was an α sat III probe) were kindly provided by Mariano Rocchi (Bari, Italy) and labeled via DOP-PCR as described by [37]. The α- satellite probes CEP 4, CEP X, CEP Y and CEP 18/X/Y to visualize centromeric regions in interphase nuclei and tissues were obtained by Vysis GmbH, Downers Groove IL, USA. The probes were labeled with the fluorophores Spectrum Aqua, Spectrum Green and Spectrum Orange (Spectrum dyes are a trademark of Vysis Corporation) as well as DAC (diethylaminocoumarine, NEN Life Science Products Inc., Boston, USA) and R110 (rhodamine 110, PE Applied Biosystems Inc., Foster City, USA). The subtelomer probes were kindly provided by Lyndal Kearney (Oxford, England). Antibodies against Digoxygenin or Biotin labeled with the fluorophore FITC were from Boehringer Mannheim, Germany. The DNA counterstains DAPI and ethidium bromide were obtained from Molecular Probes, Eugene, USA.

FISH Procedure

The principle of FISH using direct and indirect fluorophore labeled DNA probes is depicted in Fig. 3. In particular, the human chromosomes were treated with pepsin, 0.01% in 10 mM HCl, at 37°C for 5 min and fixed in 1% formaldehyde/PBS/50 mM $MgCl_2$ for 10 min at 22°C (room temperature). After dehydration in increasing ethanol series of 70%, 95% and 100% for 3 min each [10] the denaturation of the probes was performed using 70% formamide/2x SSC (saline sodium citrate), at 72°C for 3 min. A dehydration step followed as above, with cold 70% ethanol (-20°C). 10–15 µl of the detection probes were denatured as described in the protocols given by Vysis, (typically 5 min at 75°C followed by rapid cooling to 4°C). After adding to the specimens, the slide was covered with a coverslip and sealed with rubber cement, hybridization was done in a humidified chamber at 37°C for 12–15 hours. The slides were washed to remove mismatched or non-hybridized probe (rapid wash, [38]) in 0.4x SSC at 68°C for 2 min and 1 min at 22°C in 4x SSC/0.05 % Tween. Fluorophore conjugated avidin or anti-biotin antibodies are used to label biotinylated probes. Anti-digoxigenin antibodies were used alternatively for digoxigenin labeled probes. The specimens were mounted with Vectashield (Vector Laboratories Inc., Burlingame, USA) as antifading agent, covered with a 170 µm coverslips and some were counterstained with DAPI.

Tissue – FISH: Skin- and kidney- tissue was cryosectioned to slices of 10 – 12 µm and fixed on slides covered with 3-aminopropyl-triethyloxysilane (Sigma, Deisenhofen, Germany). The pepsin – treatment was for 10 min and the hybridization time was increased to 36 hours. Post-hybridization wash was performed with 50% formaldehyde/PBS for 15 min and additional in 2x SSC for 15 min. All the other steps were as described above. After probe detection the nuclei were counterstained with ethidium bromide (10 µM/PBS).

For laser microscopy of tissue, the specimen was introduced in sterile miniaturized cell chambers ("MiniCeM", JenLab GmbH, Jena, Germany) with two 0.17 mm thick chamber windows for microscopy with high NA objectives.

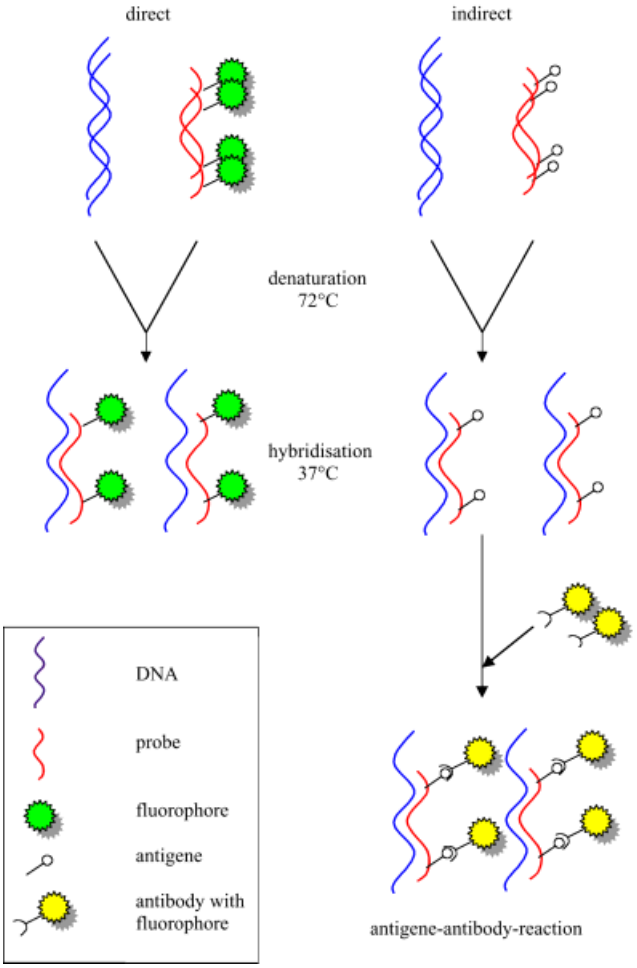


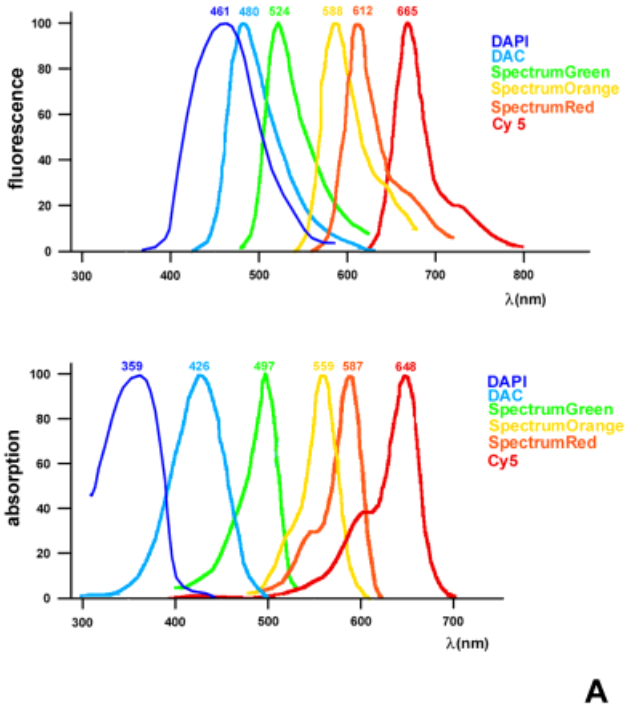
Fig. 3. *Principle of FISH with direct and indirect fluorophore-labeled DNA probes to visualize specific genomic regions.*

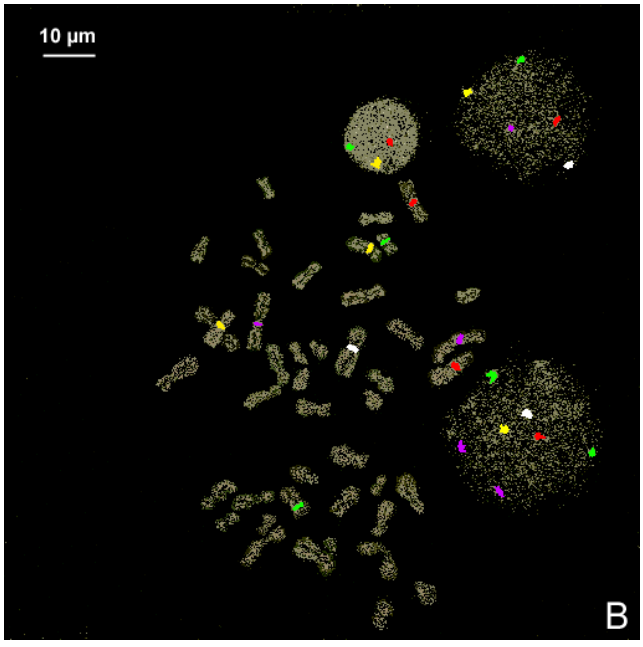
Results and Discussion

Threshold for Photodamage

Before starting the two-photon multicolor FISH experiments, the threshold for photo-induced damage by the 800 nm femtosecond laser excitation to the DNA biomolecule was determined. Using a frame rate of 0.125 s^{-1} (512 pixel x 512 pixel) and a beam dwell time on the chromosome of 8-20 ms per scan, respectively, unstained fixed human

chromosomes were scanned 20 times in dependence on the *in situ* laser power (laser power on the target). When reaching a mean *in situ* power of 30 mW severe morphological damage occurred. Raising the power up to 50 mW, the DNA material was partially removed. When performing a single line scan, the chromosome was dissected with a cut size below 500 nm.





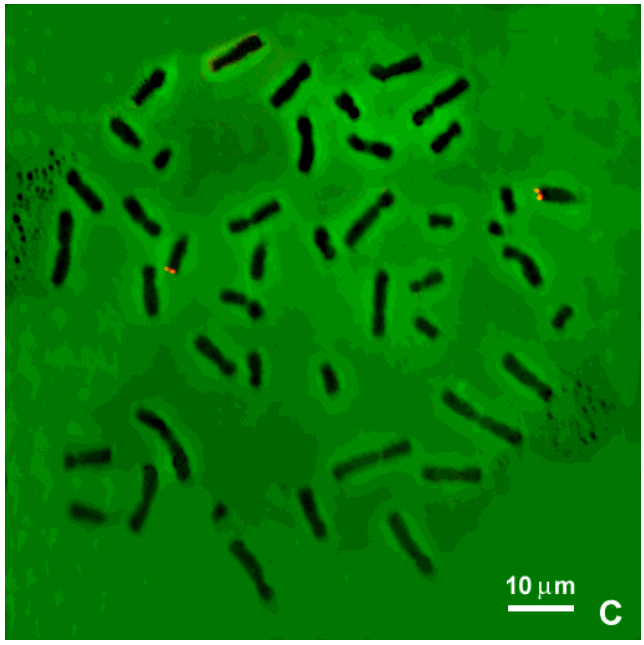


Fig. 4. A. *One-photon excitation and emission spectra of common FISH fluorophores which can be excited with intense 800 nm radiation.* **B.** *Multicolor two-photon imaging of centromeric regions in human metaphase chromosomes using the coded FISH fluorophores DAC (C-1, violet color), Spectrum Orange (C-3, red color), Spectrum Green (C-6, yellow color), R110 (C-17, green color), Spectrum Aqua (C-X, white). The unspecific chromosome fluorescence is based on the application of formaldehyde in FISH procedure.* **C.** *Imaging of sub-telomer regions of the two chromatides (C-14). The DNA probe was indirectly labeled with biotin/FITC. The fluorescence is superimposed with the phase contrast image using 800 nm transmission.*

The threshold for the onset of visible morphological photodamage during scanning was significantly reduced down to 10 mW *in situ* power in the case of application of counterstains or antifading agents to chromosomes. In contrast, the fluorescence of DAPI-labeled chromosomes could be excited via a two-photon excitation process with a minimum power of 25 µW. In the case of DAPI imaging, the laser power for non-destructive DNA imaging could be therefore varied by more than two orders of magnitude. To substantiate that the fluorescence is indeed based on a two-photon effect, we measured the fluorescence intensity by changing the incident NIR laser power. A quadratic dependence of emission signal vs. laser power was observed establishing the two-photon origin of the fluorescence.

Fluorescence Excitation of FISH Fluorophores

The FISH procedure on DNA targets, such as on human chromosomes, led to a weak unspecific blue/green fluorescence (background fluorescence) of non-hybridized

genomic regions when exposed to 800 nm femtosecond laser pulses at 5 - 7 mW laser power. The origin of this emission is based on the application of ingredients for the chromosome fixation, namely by the component formaldehyde. The fluorescence intensity was found to be dependent on incubation time of formaldehyde and was significantly reduced when the time was decreased from 10 min (standard protocol) to 5 minutes. In contrast, chromosomes of living cells in metaphase did not show any fluorescence in the visible range when excited with intense NIR radiation. The formaldehyde mediated signal to noise (S/N) ratio in chromosomes was found to be 5:1 to 10:1.

Intense NIR radiation at 4-32 µs beam dwell time per pixel was able to excite and to image a variety of FISH fluorophores at 1-7 mW mean power. When applying FISH-probes for specific chromosome regions, the background fluorescence enabled the localization of the fluorescent "dots" on the chromosomes without the application of additional counterstains. For a variety of FISH fluorophores a typical S/N ratio of FISH-fluorophore emission to background emission was found to be 10:1. The 800 nm excitation enabled the excitation of a variety of blue-emitting FISH-fluorophores (DAC and Spectrum Aqua with an emission maximum at 480 nm and one-photon excitation maxima at 426 nm and 433 nm) but also of fluorophores emitting in the green, yellow and red spectral range.

Although the one-photon absorption spectrum revealed low absorbance at 400 nm, which is half the NIR excitation wavelength, such common fluorophores like Spectrum Green (559nm/38nm one-photon excitation maximum/FWHM), Spectrum Orange (524nm/30nm), Spectrum Red (587nm/35 nm) and Cy 5 (648nm/40nm) could be excited to fluoresce (Fig. 4A). The excitation of a variety of conventional FISH-fluorophores at 800 nm provided the unique possibility of multicolor imaging of different genes and genomic regions by means of only one scan and at only one excitation wavelength.

In order to demonstrate the potential of two-photon multicolor FISH, the five centromeric probes pAL1 (0.500+0.500 kb), pAE0.68 (0.680 kb), pEDZ6 (0.340 kb), pZ17-14 (1.020 kb) and pDMX1 (2.000 kb) for the chromosomes 1, 3, 6, 17 and X, respectively, were directly linked to five different FISH fluorophores and applied to a metaphase spread of lymphocytes. Fig. 4B demonstrates the result of one scan of a metaphase spread. A weak unspecific chromosome and nuclei fluorescence and various color-coded high contrast fluorescent dots of the FISH fluorophores are shown. In particular, the FISH fluorophores Spectrum Aqua (C-X), DAC (C-1), Spectrum Green (C-6), R110 (C-17) and Spectrum Orange (C-3) which emit in the blue, green and orange spectral range, respectively, have been imaged. With an incorporation of fluorescent molecules of 1-3 % [5], the number of fluorescent molecules per probe is in the case of the smallest probe (pEDZ6) 3 to 10 and for the largest (pDMX1) 20 to 60. The α-satellite DNA in chromosome X exists as a 2.0 kb repeat unit of approximately 5,000 copies [39]. Therefore, theoretically 5000 copies of the 2.0 kb probe (pDMX1) and a maximum number of 100,000 to 300,000 fluorescent molecules could bind to the centromeric region of the 140 Mbp X chromosome. Since the centromeric region of the 110 Mbp chromosome 1 has only 190 kbp [40], the maximum number of fluorescent molecules is therefore reduced by a factor of about 50 which results in a less intense fluorescence signal. Fig. 4B shows also fluorescent hybridization dots in three nuclei of lymphocytes. Because the nuclei images represent only fluorophores in one particular focal plane, less than the expected 9 fluorescent dots per nucleus have been visualized.

Figure 4C represents imaging of small DNA probes for visualization of subtelomeric regions. The image of these regions on the two chromatides (Q arm) of chromosome 14 of a human lymphocyte was obtained after hybridization of an indirectly biotin/FITC labeled 40 kb single locus DNA probe.

Fiber-FISH

In order to demonstrate the capacity of two-photon FISH for gene detection, Spectrum Green and Spectrum Orange labeled probes for the c-abl proto-oncogene and the bcr (breakpoint cluster region) gene have been applied to a thin single fiber-DNA molecule. Whereas the gene abl is located on chromosome 9 (9q34), the gene bcr is on chromosome 22 (22q11.2). In chronic myelogenous leukemia and acute lymphotic leukemia, a translocation t(9;22)(q34;q11.2) may occur which results in a fusion of both genes (Philadelphia chromosome) [5].

The image of the Spectrum Green labeled bcr locus probe with a size of 300 kb (Fig. 5) reveals a typical "beads on a string" pattern [41] with a total length of 40 µm. Assuming that the 40 µm fluorescent region reflects the length of a probe molecule, a base density of 7.5 kbµm^{-1} can be calculated. Compared to the value of 3 kb µm^{-1} in the Watson-Crick model, the specific fiber used in the experiment is therefore condensed by a factor of 2.5. The number of fluorescent molecules (1-3% of the base number) can be estimated to range between 75 µm^{-1} and 225 µm^{-1} per strand.

The lower part of the image reflects the two-photon excited fluorescence image of the abl locus Spectrum Orange probe of 650 kb. The probe size is larger than the 225 kb abl gene and covers also the ass gene. A similar pattern of the 800 nm excited orange fluorescence was obtained, however, the condensation state appears to be higher.

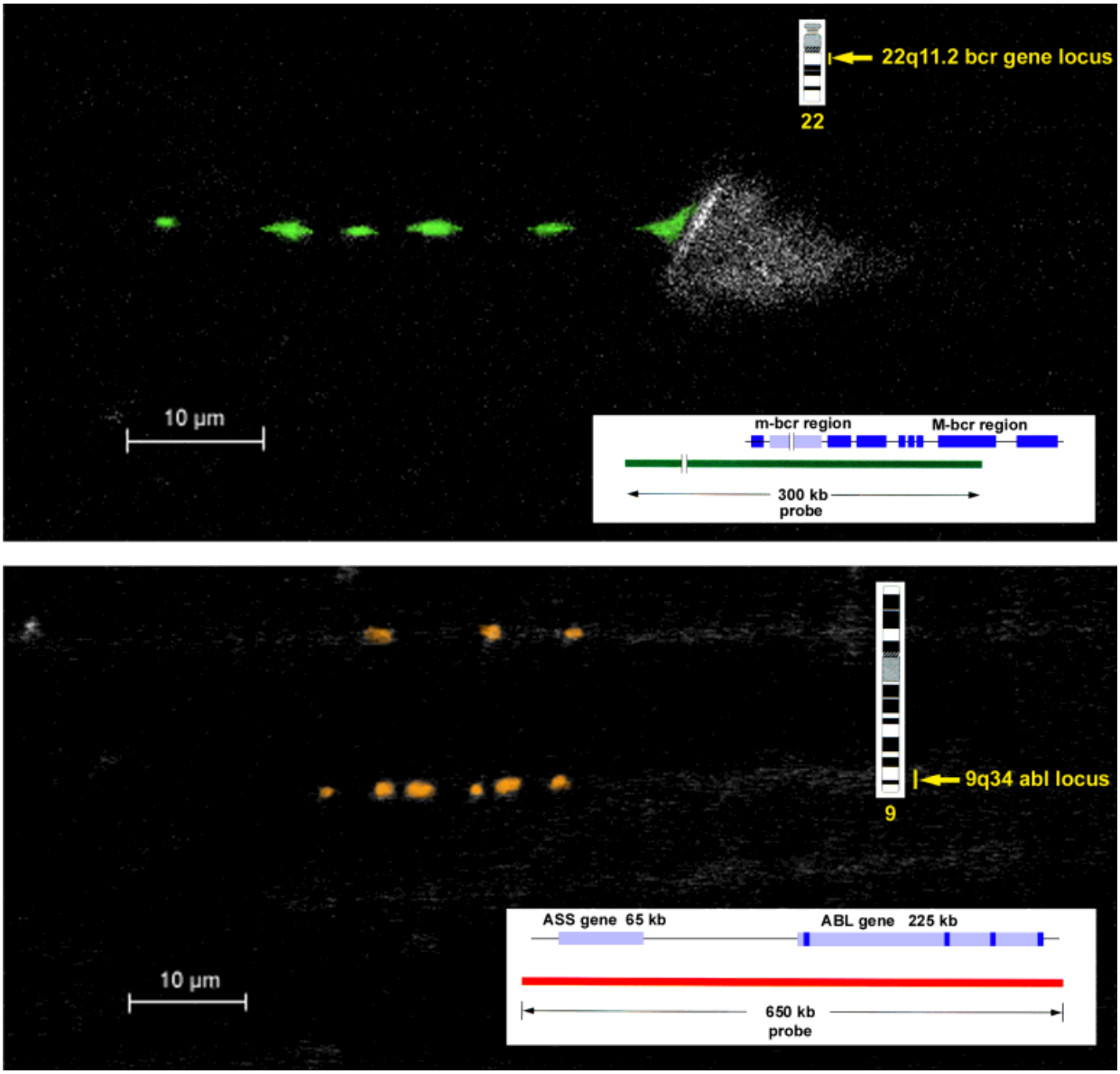


Fig. 5. *Gene imaging using fiber-DNA and schemes of genes loci. The two-photon induced Spectrum Green fluorescence indicates location of the bcr gene using a 300 kb probe, the Spectrum Orange fluorescence represents the location of the ass (argininosuccinate synthease) and abl (proto-oncogene) genes using a 650 kb probe. In the case of the bcr gene probe, a concentration of fluorescent molecules of 75-225 µm^{-1} per strand was estimated.*

Three dimensional Two-Photon Multicolor FISH

The two-photon excited fluorescence image corresponds always to a thin optical section due to the high spatial resolution in two-photon microscopes with high NA objectives. When measuring metaphase spreads with a submicron chromosome thickness (thickness of a dry spread: 100-300 nm), all hybridization signals appear in the focal plane. However when measuring interphase nuclei by a single 2D image as shown in Fig. 4B, the number of detectable FISH signals is limited. Non-resonant two-photon or three-photon fluorophore excitation with high NA objectives offers the unique intrinsic possibility of optical sectioning with submicron resolution. We used 3D two-photon Multicolor FISH to investigate amniotic fluid cells for prenatal diagnosis.

The genomic resolution in FISH is limited by the spatial resolution of the microscope and the chromatin condensation state. Whereas the resolution of metaphase chromosomes as mentioned is some megabases, the less condensation in interphase nuclei allows FISH resolution of 100 kb. Figure 6 demonstrates four-color-detection of the

fluorophores Spectrum Aqua bond to chromosome 18 centromer (white false color), Spectrum Green bound to X chromosome centromer (green false color), Spectrum Orange bound to Y chromosome centromeric region (red false color) and DAPI (blue false color). The three white hybridization signals in the stack of optical sections through the 10 µm thick cell nucleus indicates a trisomy 18 (Edwards syndrome) of a male embryo.

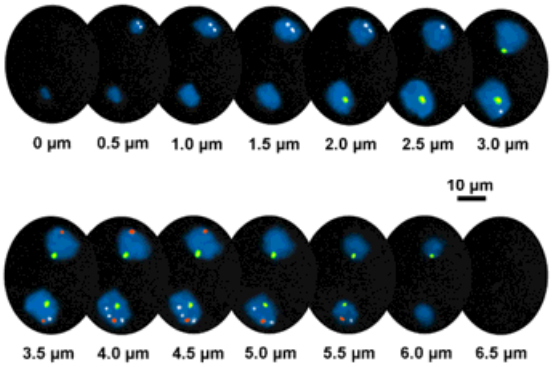


Fig. 6. *False-color coded optical sectioning (dz = 0.5 µm) and four-color-detection of nuclei of amniotic fluid cells with the 800nm excited fluorophores Spectrum Aqua (centromer chromosome 18, white), Spectrum Green (centromer X chromosome, green color), Spectrum Orange (centromer Y chromosome, red color) and the DNA counterstain DAPI (blue color). The presence of three white fluorescent dots indicate the case of a trisomy 18 (Edwards syndrome).*

FISH in Human Biopsies

800 nm exposure of cells and tissues leads to a two-photon excited autofluorescence based on the presence of endogenous fluorescent molecules such as NADH, NADPH, flavins, collagen, elastin and metal-free porphyrins. Additional background fluorescence is induced by the FISH procedure, in particular the fixation with formaldehyde.

Nevertheless, we were able to image FISH fluorophores in cryosections of kidney tissue of a male patient. The FISH signal to background fluorescence ratio varied between 6:1 and 20:1. The centromeres of three different chromosome territories were labeled with the FISH-probes Spectrum Green (C-4), Spectrum Orange (C-X) and Spectrum Aqua (C-Y). The long incubation time of 36 hours led to complete diffusion of the probes through the whole tissue.

As demonstrated in Fig. 7, twenty serial optical sections were visualized every 0.5 µm using an 40x/1.3 oil immersion objective at 7 mW mean power. The centromer signals could be detected through different layers of the specimen. One fluorescent structure was found to extend through a depth of about 3.5 µm whereas other structures measured about 1 µm. The counterstain ethidium bromide was applied to assign intracellular and intranuclear location of the centromeric regions. We were able to detect 8 nuclei in a 30x30x10 μm^3 which corresponds to a cell density of 1 cell/ picoliter. One nucleus, located in 1-8 µm tissue-depth (central part of the image), seemed to be morphologically intact and all 4 expected signals were detected. Other nuclei did not show all signals because of the cutting procedure.

Discussion and Perspectives

We developed a single molecule technology for imaging specific multiple fluorophore labeled regions of the DNA macromolecule (e.g. genes) with submicron spatial resolution based on a sub-femtoliter fluorescence excitation volume and two-photon effects by intense femtosecond laser pulses at 800 nm. The tiny excitation volume due to the nonlinear dependence of fluorescence signal vs. laser intensity as well as the use of high NA focusing optics excluded problems with out-of-focus photodamage, out-of-focus background fluorescence and expensive and less efficient fluorescence photon collection units. It provides the intrinsic possibility of pinhole-free 3D FISH fluorophore imaging. Within a certain window of MW/cm^2 and GW/cm^2 laser intensities, nondestructive two-photon fluorescence imaging of genomic regions could be performed. However, intensities in the TW/cm^2 range led to highly-localized plasma-induced DNA destruction which can be used for nanoprocessing of DNA molecules [42].

DNA probes of 0.3-2 kb size labeled with common blue-, green-, yellow- and red-emitting FISH fluorophores with an incorporation of 1- 3 % [5] that target repetitive elements within the centromer of individual chromosomes and DNA counterstains have been visualized by simultaneous absorption of two low-energy photons although no fluorophore possesses an one-photon absorption maximum at half the main excitation wavelength. Multifluorophore excitation with only one excitation wavelength simplifies Multi-Gene-Imaging. The two-photon excited formamide-based unspecific fluorescence may replace the use of counterstains in some experiments and the large spectral shift between 800 nm excitation and visible fluorescence excluded the separation problems associated with conventional FISH studies.

Using the unique high light penetration depth in the NIR spectral range of typically 3 - 6 mm in most tissues [20], 3D two-photon Multicolor FISH can be applied to study the architecture of the biomolecules RNA and DNA in biopsies [43-45], embryos [13,46] and –in perspective- also in living cells and tissues [13] with submicron resolution. Two-Photon Multicolor FISH as single molecule technique provides therefore a novel tool in the field of *in situ* molecular diagnostics, in particular for molecular pathology, molecular cytogenetics and developmental biology.

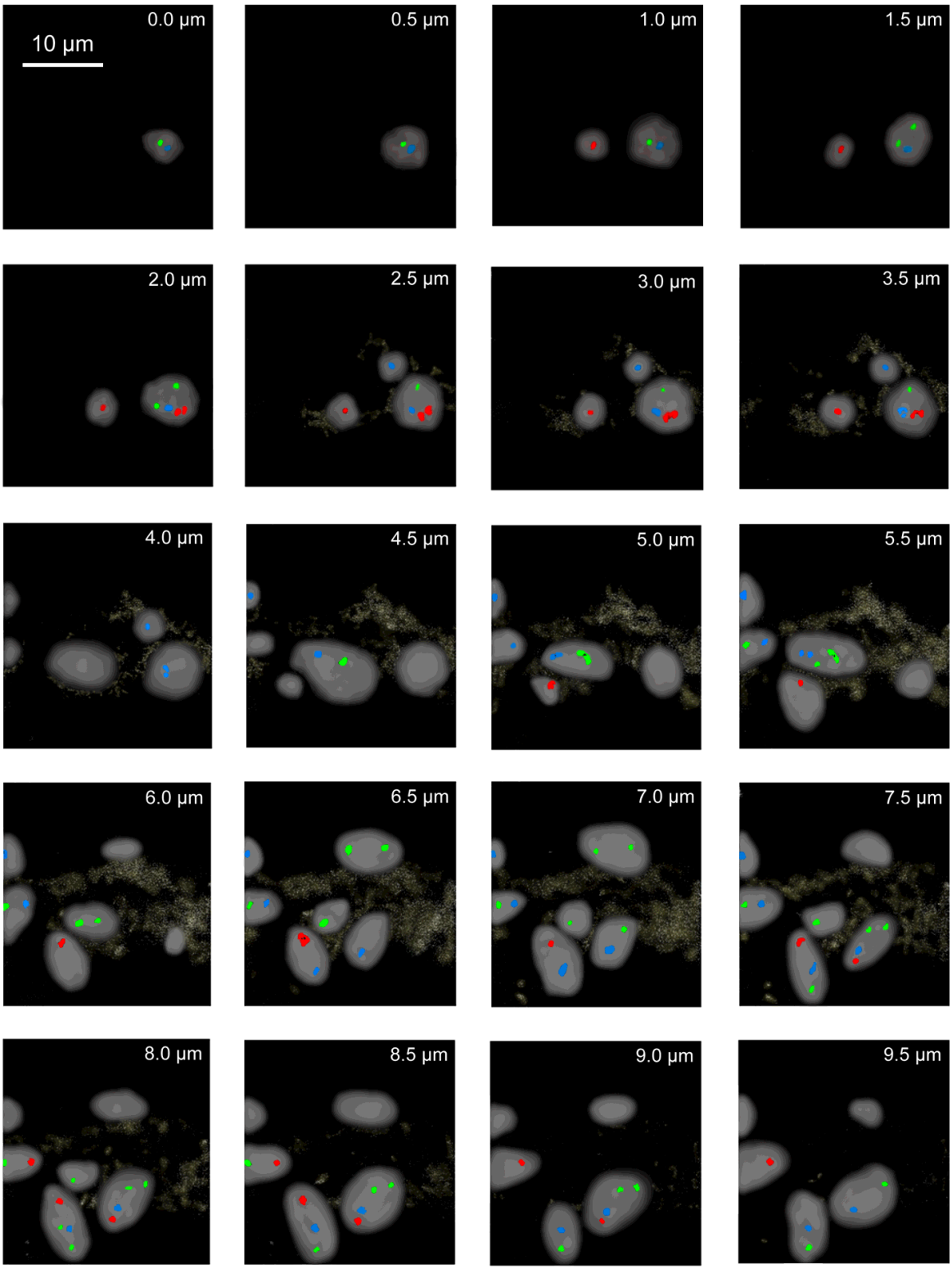


Fig. 7. *Imaging of centromeric fluorescent probes in human biopsies by three dimensional two-photon multicolor FISH. The localization of Spectrum Green (green color) labeled C-4 probes, Spectrum Orange (red color) labeled C-X probes and Spectrum Aqua (blue color) labeled C-Y probes are depicted in a 10 µm thick kidney cryosection. The nuclear area was imaged after counterstaining with ethidium bromide.*

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