

Reports

Miniaturized FISH for screening of onco-hematological malignancies

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Fluorescence in situ hybridization (FISH) represents a major step in the analysis of chromosomal aberrations in cancer. It allows the precise detection of specific rearrangements, both for diagnostic and prognostic purposes. Here we present a miniaturized FISH method performed on fresh and fixed hematological samples. This procedure has been developed together with a microfluidic device that integrates cluster-assembled nanostructured TiO₂ (ns-TiO₂) as a nanomaterial promoting hematopoietic cell immobilization in conditions of shear stress. As a result of miniaturization, FISH can be performed with at least a 10-fold reduction in probe usage and minimal cell requirements, creating the possibility of using FISH in genetic screening applications. We developed the protocol on tumor cells and bone marrow (BM) from a normal donor using commercially sex-specific and onco-hematology probes. The procedure was then validated using either BM or peripheral blood (PB) from six patients with hematological diseases, each associated with different genetic lesions. Miniaturized FISH demonstrated comparable performance to standard FISH, indicating that it is suitable for genetic screenings, in research, and in clinical settings for the diagnosis of samples from onco-hematological malignancies.

Introduction

Considerable effort is currently being concentrated toward the development of analytical tools with improved performance, sensitivity, and information throughput through integrated approaches of engineering and miniaturization at affordable costs (1,2); new disciplines such as microfluidics, biotechnology, and nanotechnology, are expected to play a key role in driving this innovation (3).

Among analytical assays based on fluorescence read-out, fluorescence in situ hybridization (FISH) is a widespread and informative tool, utilized in both basic research and in diagnostics (4,5). Through fluorescence detection by hybridization with DNA probes of chromosomal sequences in fixed nuclei on slides, FISH represents a robust method able to resolve complex genetic rearrangements that would have remained unresolved by conventional cytogenetics, relying on the analysis of chromosome structure in metaphase. This consideration particularly applies to tumors showing poor chromosome morphology in metaphase preparations, therefore

preventing accurate diagnostic evaluation. In fact, using appropriate probes, FISH can also be successfully performed on interphase nuclei to detect specific chromosomal rearrangements, such as the 9/22 translocation in chronic myeloid leukemia (6), offering an essential tool for cancer diagnosis and prognosis.

FISH technology has remained substantially unchanged since its introduction approximately 20 years ago (7,8), and its widespread utilization was mainly hampered by its cost. Indeed, in many laboratories, FISH is only utilized as a second step on a selected number of cases previously screened using classical cytogenetic techniques. Additionally, this technique may suffer severe limitations whenever samples with poor cell content have to be managed.

To reduce costs and improve assay performance, microfluidics (9,10) can provide a means for miniaturization through the engineering of polymeric microchannels in devices wherein reagents can be loaded in small volumes, and cellular samples can therefore be concentrated. However, relevant technical challenges have to be overcome: due to the micrometric section of channels

in such tools, flowing fluids cause intense shear stress on cells. This can cause them to be easily disrupted or detached, which can therefore compromise the assay (11).

In this context, miniaturization of FISH through microfluidic methods could be a promising solution only if cell immobilization inside the microchannel is appropriately provided.

Sieben and coworkers recently proposed a “lab-on-a-chip” approach for FISH miniaturization based on microfluidic technology (12,13). They engineered a fully integrated chip in which hematopoietic cells were immobilized by heating inside a microwell together with an automated FISH protocol; however, the complexity of the chip (which required a sophisticated fabrication facility), and its consequent cost were indicated by the authors as key challenges that needed to be addressed in order to make this technique accessible.

We have focused our research on the characterization of biomaterials and coatings with properties promoting cell adhesion (14), and discovered that the cluster-assembled nanostructured TiO₂ coating (ns-TiO₂) is able to trigger a rapid and efficient immobilization of both living and fixed hematopoietic

cells, even in the presence of prolonged shear stress (unpublished data). To fully exploit this feature, we have engineered a simple device, based on microfluidics, to set up a miniaturized FISH approach. The device consists of a polymeric microfluidic pad, with a single straight microchannel, adhering to a standard glass slide coated with ns-TiO₂ that enables rapid immobilization of cells in a small and confined space.

The efficiency of the approach was tested by performing FISH on a panel of cultured hematopoietic tumor cells as well as on bone marrow (BM) from normal donor, prepared from fresh samples. Its performance versus the standard FISH protocol was also evaluated in either BM or peripheral blood (PB) of different cases of hematological malignancies, such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), and a case of sex chromosome chimerism after BM transplantation.

By comparing classic versus miniaturized FISH, we obtained a similar degree of accuracy, quality, and reproducibility with respect to the standard protocol. The procedure is simple, and the analysis is performed on a standard fluorescence microscope both at low and high resolution. In addition, automation of the procedure can be envisaged when genetic screening programs are planned.

Materials and methods

Microfluidic device

The microfluidic device consists in a microfluidic pad (Figure 1A) made of polydimethylsiloxane (PDMS, Sylgard 184; Dow Corning, Midland, MI, USA) at a 10:1 ratio of Curing Agent with a straight microchannel; the PDMS microfluidic pad has been manufactured according to standard replica molding procedures (15).

The 10 × 0.3 × 0.05 mm mold feature defining the microchannel was carefully machined to avoid the formation of surface defects and keep roughness well below the micrometric scale on the top of the PDMS microchannel. This limited light scattering allowed microscopical inspection of the cellular sample through the microchannel and prevented the formation of bubbles during the steps of the FISH protocol.

The microchannel has a volume of 0.15 μL and a bottom of ns-TiO₂-coated surface of 3 mm². Two ports (wells) connect the microchannel with the upper surface for reagent loading (IN well, 1.2-mm diameter) and aspiration (OUT well, 0.7-mm diameter).

The available area on the coated surface of the microchannel was a compromise between miniaturization and the need to accommodate a statistically meaningful number of cells for the FISH assay (≥1000 cells).

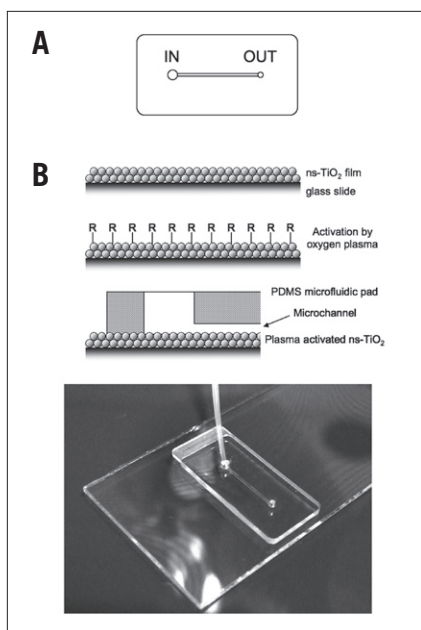


Figure 1. Microfluidic device for miniaturized FISH. (A) Sketch of the microfluidic PDMS pad. The pad has a size of 20 × 10 × 1 mm, and the microchannel is 1 cm long, 300 μm wide, and 50 μm deep. Two wells connect the microchannel with the top surface: an IN well (1.2-mm diameter) for reagent loading and an OUT well (0.7-mm diameter) for aspiration. (B) Complete structure of the FISH device (vertical section view). Glass slides are functionalized with ns-TiO₂, then treated with oxygen plasma to increase wettability (R means chemisorbed oxygen radicals); finally, the PDMS microfluidic pad is assembled on the slide, by spontaneous adhesion. An exemplary picture of a FISH device is presented; a 10-μL tip is used for cell loading.

The microfluidic pad was manually assembled on top of a glass slide (see Figure 1B) (Nexterion glass D, cleanroom-cleaned; SCHOTT AG, Mainz, Germany) previously coated with 50 nm ns-TiO₂. A detailed description of the coating method and of the principles of operation of nanoparticle sources can be found in References 16 and 17. After deposition, ns-TiO₂-coated slides were exposed to oxygen plasma (Colibri, Gambetti, Italy) for 150 s at 100 W to increase wettability and vacuum-stored (AmpliVac Mobile; Genewave, Palaiseau, France) until use, before assembling with the PDMS pad.

Although the adhesion strength of the microfluidic pad was high enough to prevent its detachment during the FISH protocol, the adhesion was reversible, allowing the pad to be easily removed at the end of the protocol for subsequent imaging.

Cells and culture conditions

Human cells lines Daudi (Burkitt lymphoma), Jurkat (T-cell acute leukemia), NB4 (acute promyelocytic leukemia), Raji (Burkitt lymphoma), and U937 (histiocytic lymphoma) were cultured in RPMI-1640

medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (Gibco, Invitrogen, Carlsbad, CA, USA), 1% penicillin streptomycin solution (Cambrex, East Rutherford, NJ, USA), and 1% L-glutamine (Cambrex); KG1a (acute lymphoblastic leukemia) cells were cultured in IMDM medium (Lonza) supplemented with 20% FBS (Gibco, Invitrogen), 1% penicillin streptomycin solution (Cambrex), and 1% L-glutamine (Cambrex). All cell lines were provided by IEO, Milan, Italy, grown in tissue culture flasks (Falcon; BD Biosciences, San Jose, CA, USA) at 37°C in a humidified incubator with 5% CO₂, and split regularly to maintain exponential growth.

Human BM and PB from normal donor or patients were provided—respecting ethical regulations—by IEO, collected in heparin or EDTA, and used after 0–5 days of storage at room temperature (RT).

Miniaturized and standard FISH

Cultured cells (1 mL exponentially growing cells) were placed in a 1.5-mL tube and washed three times with 1× Dulbecco's PBS (DPBS) by centrifuging in a microcentrifuge at 500×g for 5 min, counted, and resuspended at a concentration of 8000–15,000 cells/μL.

Aliquots of BM (100–500 μL) or PB (0.5–1 mL) of normal donor or patient cells were treated with red blood lysis (RBL) buffer (0.15 M NH₄Cl, 9.93 mM KHCO₃, 0.13 mM EDTA; Sigma-Aldrich, St. Louis, MO, USA) to a volume of 10 mL, kept for 5 min at 4°C, then centrifuged at 425×g for 5 min. Supernatant was discarded, and cells were resuspended again in 10 mL RBL buffer, then centrifuged at 600×g for 5 min.

Cells were resuspended in 1 mL 1× DPBS and transferred to a 1.5-mL tube, washed twice in 1× DPBS by centrifuging at 500×g, and resuspended at the concentration of 15,000–25,000 cells/μL. Before use, microfluidic devices were preincubated at 37°C on top of a hot plate (for ≥2 min). Then 1.5 μL cell suspension was pipetted into the IN well and left to enter the channel by capillarity; the device was then incubated at 37°C for 4 min to allow cell adhesion. Then, 20 μL Carnoy's fixative (3:1 methanol:acetic acid; Carlo Erba, Milan, Italy) was added and left to diffuse into the channel to fix the cells for 2 min and then completely aspirated. All the following reagents were loaded by dispensing a droplet over the IN well and aspirating it from the OUT well with a syringe pump at 5.5 μL/s (KDS120; KD Scientific, Holliston, MA, USA); cell adhesion was strong, as vacuum pump aspiration was also adopted with no occurrence of cell loss.

After cell fixation in the microchannel, the following series of reagents were used, while incubations at different temperatures were

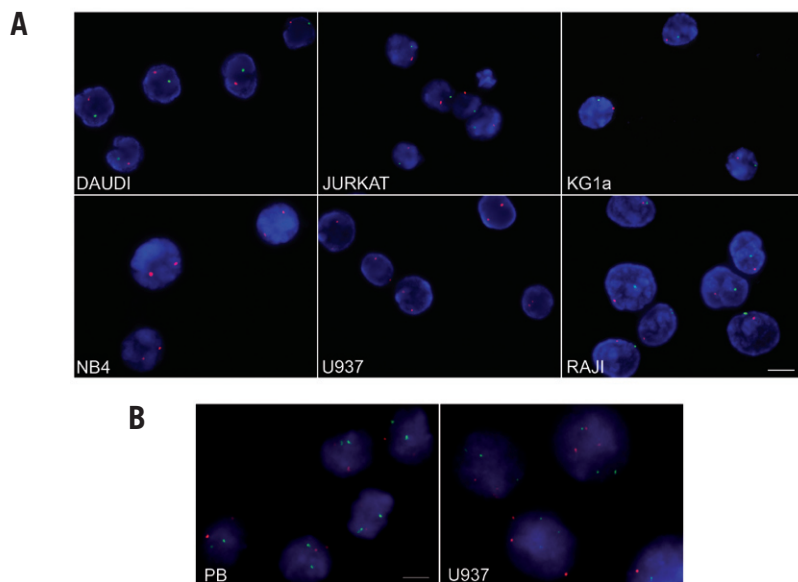


Figure 2. Miniaturized FISH with CEP and LSI probes on tumor cells. (A) Panel of tumor cells with CEP probes for sex chromosomes. Images from representative cells are acquired at 40 \times objective magnification. Red signal represents chromosome X; green signal represents chromosome Y; scale bar, 10 μ m. (B) PB from normal donor (control cells) and U937 cells analyzed with LSI AML1/ETO probe. Images from representative cells are acquired at 100 \times objective magnification. Red signal represents chromosome 8, while green signal represents chromosome 21; U937 cells showed trisomy for both chromosomes; scale bar, 4 μ m.

performed on hot plates: 2 \times saline-sodium citrate buffer (SSC) for 15 min at 37 $^{\circ}$ C; digestion buffer 0.005% pepsin in 0.01 N HCl (Sigma-Aldrich) for 10 min at 37 $^{\circ}$ C; 1 \times DPBS for 5 min at RT; postfixative 50 mM MgCl₂, 0.95% formaldehyde (Sigma-Aldrich) in 1 \times DPBS for 5 min at RT; 1 \times DPBS for 5 min at RT; EtOH 70%, 85%, and 100% (BDH, VWR International, West Chester, PA, USA) for 1 min each at RT; denaturing solution (70% formamide in 2 \times SSC; Sigma-Aldrich) for 3 min at 75 $^{\circ}$ C; and EtOH 70%, 85%, and 100% for 1 min each at RT. After EtOH 100% aspiration, slides were left to dry completely at 60 $^{\circ}$ C for 2 min and then loaded with 0.3 μ L denatured probes (see list). Microchannel wells were sealed with a drop of mineral oil (Sigma-Aldrich) to prevent evaporation and incubated overnight at 37 $^{\circ}$ C. After incubation, PDMS pads were removed, and slides were dipped in coplin jars containing prewarmed wash solution A [0.3% Nonidet-P40 (NP40; Sigma-Aldrich) in 0.4 \times SSC] for 2 min at 73 $^{\circ}$ C and wash solution B (0.1% NP40 in 2 \times SSC) for 1 min at RT, air-dried, and mounted with DAPI II (Abbott Molecular, Des Plaines, IL, USA) for microscopy analysis.

The cytogenetic pellet in Carnoy's fixative for standard FISH was prepared following standard procedures and processed according to each probe's data sheet.

For the use of cytogenetic pellets with the microfluidic device for miniaturized FISH, see the Supplementary Materials.

For sensitivity and specificity analysis and for result comparison with standard FISH, signals were evaluated by analyzing 200 cells using an Olympus BX61 microscope (equipped with a F-View II camera) (Olympus Europa GmbH, Hamburg, Germany) with an UPlanApo 100 \times immersion objective (N.A. 1.40) and specific filters (Olympus U-MNIBA3 FITC, U-MWIG3 Cy3, U-MNUA2 DAPI), taking images of representative cells. Images were acquired using CellA software (Olympus Europa GmbH) and color-merged with Adobe Photoshop 7.0 (Adobe Systems, Inc.; San Jose, CA, USA). We evaluated the performance quality of miniaturized FISH versus the standard method by means of the coefficient of variation (CV) weighted by the average value between the percentage of positivity for a given genetic lesion for each patient for both methods.

CV < 10% means that there is an equivalent level of performance of the test method compared with the reference method.

FISH probes

For FISH experiments, the following probes were used. The probe used for sex chromosome detection was CEP X Spectrum Orange/Y SpectrumGreen Direct Labeled Fluorescent DNA probe kit (Abbott Molecular). Onco-hematological probes were Vysis Locus Specific Identifier (LSI) p53/LSI ATM and LSI D13S319/LSI 13q34/CEP 12 Multi-Color probe; Vysis LSIBCR-ABL Dual Color, Dual Fusion Translocation probe; Vysis LSI

IGH Dual Color, Break Apart Rearrangement probe; Vysis LSI IGH/CCND1 Dual Color, Dual Fusion Translocation probe; Vysis LSI AML1/ETO Dual Color, Dual Fusion Translocation probe; Vysis LSI CBFb Dual Color, Break Apart Rearrangement probe; Vysis LSI D13S319 Spectrum Orange (all, Abbott Molecular); and Poseidon Repeat Free 6q21 & SEM 6 Control probe (Kreatech Diagnostic, Amsterdam, The Netherlands).

Results and discussion

Miniaturization of analytical assays through microfluidics represents an efficient and valid approach with uses in many different applications of biology (18). We have developed and validated a miniaturized approach for FISH with the specific aim of improving the assay for genetic screenings of onco-hematological diseases.

To test the specific performance of the assay, we performed a series of experiments with a panel of probes for different purposes.

We started using different commercial probes on either cultured tumor cells of hematopoietic derivation or normal donor samples, performing parallel experiments using either fresh cell suspension or a cytogenetic pellet; since similar results have been obtained (see Supplementary materials), we developed the method using fresh cellular samples.

Briefly, 1.5 μ L hematopoietic cultured cells (see the "Materials and methods" section for the list) were resuspended at 10,000 cells/ μ L in DPBS (yielding ~1400–1500 cells immobilized on the surface) and loaded in triplicate (three different pads) inside the microchannel, incubated for 4 min at 37 $^{\circ}$ C to permit efficient cell adhesion, then fixed in methanol/acetic acid (see details in the "Materials and methods" section).

Immobilized cells were first treated with 2 \times SSC, then pepsin, and then washed in DPBS, fixed again with formaldehyde, and finally dehydrated with a series of ethanol concentrations (70%, 85%, and 100%). DNA denaturation was achieved by pipetting a solution of 70% formamide and incubating the device at 75 $^{\circ}$ C for 3 min. Samples were then dehydrated in alcohol as before and dried at 60 $^{\circ}$ C. The hybridization was performed by adding 0.3 μ L denatured probe to the microchannel and left overnight at 37 $^{\circ}$ C, ensuring accurate sealing of the microchannel wells with a drop of mineral oil to avoid probe evaporation. The following day, PDMS pads were removed, and slides were washed in coplin jars, according to the manufacturer's recommendation.

Figure 2A summarizes the results obtained using CEP XY sex chromosome-specific probes. At least 200 nuclei were scored by

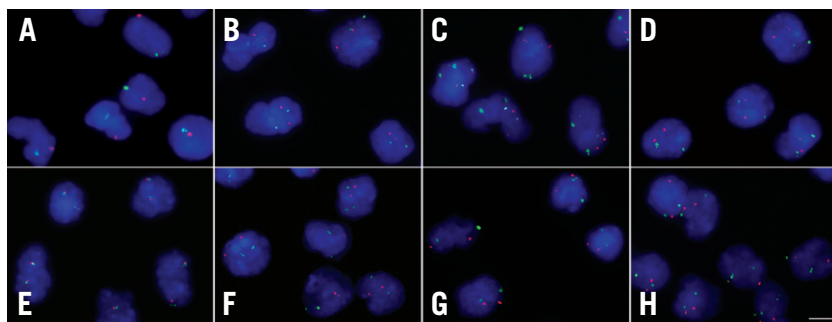


Figure 3. Validation of miniaturized FISH on hematopoietic cells from normal donor. Miniaturized FISH analysis on hematopoietic cells from BM of normal donor with a panel of onco-hematology probes (Abbott Molecular, unless otherwise indicated): (A) Vysis CEP X SpectrumOrange/YSpectrumGreen Direct labeled fluorescent DNA probe kit; (B) Vysis LSIp53/ATM; (C) Vysis LSI D13S319/LSI 13q34/CEP 12 Multicolor probe; (D) Vysis LSI BCR/ABL Dual Color Dual Fusion Translocation probe; (E) Vysis LSI IGH Dual Color Break Apart Rearrangement probe; (F) Vysis LSI IGH/CCDN1 Dual Color Dual Fusion Translocation probe; (G) Poseidon Repeat Free 6q21 & SEM 6 Control probe (Kreatech); and (H) Vysis LSI AML1/ETO Dual Color, Dual Fusion Translocation probe. Representative images were acquired at 100 \times oil objective magnification. Scale bar, 5 μ m.

automated microscopy for each cell line: 97.9% of nuclei showed clear fluorescent hybridization signals, and 95.7% of positive cells displayed, in each nucleus, the number of dots expected from their genotype (figures are the average of results on all cell lines; see Supplementary Table S1 for detailed analysis); no gross genetic alterations were found for sex chromosomes in these cultured cell lines. CEP probes are intrinsically efficient in hybridization. Therefore, to challenge the miniaturized method with LSI probes, which are usually less efficiently hybridized, we tested U937 cells with LSI AML1-ETO probe; these cells were previously characterized for molecular cytogenetic analysis and karyotype (19) and shown to present three chromosomes 8 and 21. We then compared the results of the miniaturized FISH between the aberrant U937 cells with control cells (PB) from normal donor and observed that in the U937 cells, the AML1-ETO probe detected three signals for both chromosomes 8 and 21 in 96% of the cell population, while in the control cells, the signals were normal in 97% of cells (Figure 2B). Fluorescence background was negligible and did not affect the dot count. Additionally, because of the specific design of the device that concentrates the cells in a small area, the time for image acquisition was relatively rapid: >200 cells per sample had been analyzed in <8 min at both at low and high magnification, using three different filters for the relative fluorophores. A parallel analysis was conducted on a standard FISH preparation. In this case, using automated microscopy with the same settings, 200 cells were acquired in approximately 40 min.

The approach was further validated on BM cells from normal donor using a panel of onco-hematological probes (Figure 3). After red blood cell lysis and a series of washes in DPBS, cells were counted and resuspended

in an appropriate volume of DPBS, taking into account the non-negligible smaller cell size (\sim 5- μ m diameter) of the hematopoietic cells compared with the cultured tumor cells. We adjusted the cell suspension to \sim 15,000–25,000 cells/ μ L to obtain a range of 2300–3700 cells per microchannel. After cell adhesion (three microchannels per donor), slides underwent digestion, postfixation, denaturation, and dehydration as described in the “Materials and methods” section. Then, 0.3 μ L denatured probe was loaded on microfluidic channels, and slides were incubated overnight at 37°C. Examples of these FISH experiments are reported in Figure 3 (100 \times magnification). A detailed analysis (200 cells scored per probe per patient) showed that an average of 97.8% of cells were clearly positive; in 96.2% of nuclei, the signals were scored with a normal genotype (see Supplementary Table S2 for detailed analysis).

In order to further verify the new approach in a context of hematological disease, we have evaluated in parallel, by standard and miniaturized FISH approaches, different patients with hematological malignancies characterized by specific genetic lesions.

Chromosomal aberrations are not an infrequent occurrence in tumors, and its precise detection, especially at low percentages, is a demanding task. Therefore the sex-chromosome chimerism presented on the first sample (BM case), which was the consequence of BM transplantation (female donor on a male patient), can be very appropriate for comparative efficiency analysis. The second case was a CLL patient already shown to bear chromosome 12 trisomy in a percentage of cells. Then we evaluated PB cells for two cases of CLL bearing p53, ATM, and D13S319 deletion; a case of AML for chromosome 16 inversion; and a case of CML for BCR-ABL translocation.

Probes for all genetic lesions detected are reported in detail in the “Materials and methods” section. The results are summarized in Figure 4: panels A–D and I–N report the results of miniaturized FISH, while panels E–H and O–R report the results from standard FISH protocol. At high magnification, images show representative nuclei in which we detected the specific genetic lesions (Figure 4; white arrows).

Detailed analysis of the percentage of positivity for the XY chimerism, chromosome 12 trisomy, or the specific genetic lesions is reported in Supplementary Table S3. We evaluated the CV between the two analyses and obtained low values ranging 1.6–7.5%, demonstrating the equivalence of the two approaches in terms of performance.

In addition to quantitative data, we also compared the raw black-and-white images taken by the charge-coupled device (CCD) camera, evaluating by visual inspection the signal quality and paying special attention to the signal-to-noise ratio. An example is shown in Figure 4: a comparison of green, orange, and aqua channels between panels C and G shows the neat and sharp appearance of specific signals in miniaturized FISH.

Our parallel experiments indicate that miniaturized FISH achieved the same degree of accuracy and reproducibility as standard FISH.

The device design was aimed at providing significant advantages in terms of decreasing the cost of FISH by reducing the volume of reagents and fluorescent probes (10- to 30-fold less probe compared with standard protocols); moreover, the miniaturization of the procedure enables an increase in throughput, particularly regarding the parallel processing of samples and the evaluation of the results by automated microscopy. Finally, the possibility of concentrating and collecting, in a confined space, samples with poor cell content provides the opportunity for FISH analysis of rare cells.

For instance, in the case of multiple myeloma diagnosis, cellular enrichment by cell sorting or purification (20) is necessary to obtain homogeneous populations of plasma cells; however, in many cases, the number of cells is not adequate for standard FISH, since the preparation of a cytogenetic pellet can result in massive cellular loss.

As an alternative, in miniaturized FISH enriched living cells resuspended in a small volume of buffer (\sim 1 μ L) are directly immobilized in the microfluidic channel, thus avoiding cellular loss and providing a tool for the analysis of scarce patient samples.

We therefore plan to extend the application of miniaturized FISH to the detection of other genetic diseases such as bladder or cervical cancer (21,22), where screening strategies can be envisioned.

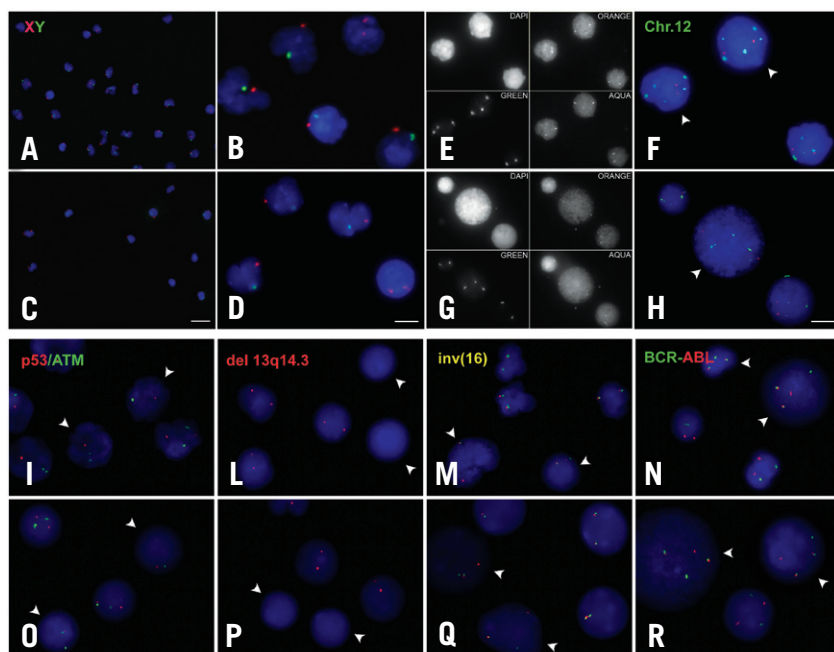


Figure 4. Comparative FISH analysis: miniaturized FISH versus standard FISH. (Upper panel) Miniaturized FISH (A–D) and standard FISH (E–H) for sex chromosome chimerism detection (A, B, E, and F) and in a CLL case (C, D, G, and H) with chromosome 12 trisomy, respectively. (A and E) Merged images at 20× objective magnification; scale bar, 20 μm. (B and F) Merged images at 100× oil objective magnification; scale bar, 5 μm. (C and G) Raw images at 100× oil objective magnification representing single channels of the different fluorescent probes (orange, chr.13q14.3; green, chr. 12p11.1-q11; aqua, chr.13q34). (D and H) Merge of different channels in panels C and G, respectively. Scale bar, 5 μm. (Lower panel) Miniaturized FISH (I–N) and standard FISH (O–R): genetic lesions detected are p53/ATM deletion in a CLL case (I and O), 13q14.3 deletion in a CLL case (L and P), chromosome 16 inversion in an AML case (M and Q), and BCR-ABL translocation in a CML case (N and R). Images are acquired at 100× oil objective magnification. White arrows on each image indicate nuclei with the relative specific genetic lesion detected.

In conclusion, our approach yields several improvements when compared to standard FISH protocol, while preserving the same level of quality: (i) the assay cost has been substantially reduced by decreasing the amount of probe required; (ii) rare cells can be processed and evaluated; (iii) the protocol is suitable for automation and increased throughput; and (iv) the time for automated fluorescence analysis of samples has been dramatically reduced.

Therefore, it can be considered an ideal tool both in basic research and in disease management, particularly suited for large genetic screenings of onco-hematological disease.

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Competing interests

A.Z., D.B., and R.C. are currently employees of Tethis S.R.L.; and E.B. and P.M. are shareholders of Tethis S.R.L.

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