microFIND® Approach to Fluorescent in Situ Hybridization (FISH)

Andrea Zanardi, Emanuele Barborini, and Roberta Carbone

Abstract

FISH technology has gained increasing attention in the management of cancer disease, either for predictive or prognostic indications. Molecular cytogenetics has greatly improved diagnostic capability of classical cytogenetics analysis of metaphase-based chromosome for the identification of genetic aberrations. The availability of a large number of fluorescent probes, each specific for different genetic lesions, together with a robust protocol for interphase FISH, provide the pathologist with the essential tools for an accurate evaluation of patient’s disease. Hemato-oncological and many of the solid tumors have been comprehensively characterized by peculiar genetic defects and are now routinely evaluated by interphase FISH. Despite the reliability of the method, which has undergone only minor changes since the 1970s, FISH assay is still hampered by reagents cost, preventing its adoption in large-scale oncological screening. In this chapter we describe a major improvement of interphase FISH assay for cytological samples through the description of the miniaturized device microFIND® that offers, besides reduction of cost per assay, a completely novel vision to the FISH technology, thanks to the perspective of full automation of FISH assay using a dedicated robotic platform for microFIND® handling, (not presently described in the chapter).

Key words: FISH, Screening, Miniaturization, Hemato-oncological cancer, microFIND®, Automation

1. Introduction

FISH technology (1, 2) relies on a series of manual procedures on biological samples that must be performed by skilled technicians in order to obtain high quality results for unambiguous data interpretations.

Among analytical assays FISH has not been substantially changed since its introduction (3): some procedures for automation have been proposed and implemented (4) to facilitate manual analysis; however the assay is costly and suffers the main drawback of not being easily scalable for disease screenings.
We have recently discovered a peculiar property of a nanotech coating (5, 6) that strongly immobilizes living and fixed, not otherwise spontaneously adhering, cells and by appropriate integration with a microfluidic element we have developed a diagnostic device, microFIND®, for FISH analysis on cytological samples.

The device is composed of a glass slide with a nanotech coating, and a polymeric microfluidic pad (7) with a microchannel and two wells for loading and aspiration of reagents. The microfluidic pad is coupled to the slide in order to have the slide constituting the bottom surface of the microchannel. Cells are loaded in the microchannel and allowed to adhere on bottom surface (of the coated slide). Due to the strong adhesion on the nanotech coating the analytical protocol can be carried out without loosening of cells, despite the significant shear stress caused by moving fluids inside the microchannel.

Following this approach, FISH can be performed in a miniaturized setting, strongly reducing sample requirement, reagents and probe volume. With dedicated automation, microFIND® provides an affordable and scalable method for genetic based-cancer screenings.

In this method we describe the device and a protocol for FISH on bone marrow and peripheral blood cells for the evaluation of a common type of leukemia, the chronic lymphocytic leukemia (CLL).

Specifically two genetic abnormalities have been tested for:

1. Deletion of locus D13S25 at 13q14.3 region that is one of the most common abnormalities in CLL. These patients have the best prognosis and most of them do not need therapy.
2. Trisomy 12, an additional chromosome 12, which is a relatively frequent finding, occurring in 20–25% of patients, indicating an intermediate prognosis.

## 2. Materials

### 2.1. microFIND® Device

1. microFIND® device is composed of two parts: a glass slide partially functionalized with a nanotech coating and a polymeric pad with microfluidic circuitry constituted by a straight channel acting as small volume reaction chamber and by two access wells (Tethis S.p.A., Milano, Italy). A larger diameter well within a cone-shaped pit is dedicated to sample/reagent loading (IN well), while smaller diameter well is dedicated to aspiration (OUT well).

2. Pads are contained in a plastic “assembler” designed to both store the pad in a proper way and prepare the final device (see Subheading 3 and Fig. 1).
3. Sample and reagents loading: standard pipettes with 50 and 10 μl tips.

4. Sample aspiration: a 10 ml syringe equipped with dedicated adaptor composed of 10 cm tube and sucker (Tethis S.p.A) is required for liquid removal.

5. As alternative for liquid aspiration the use of a motorized syringe equipped with a 20 ml syringe with a 1.5 mm tube is also possible (KDs 120, KD Scientific, Inc., Holliston, MA, USA).

2.2. Samples

1. Peripheral blood (PB) or bone marrow (BM) from a donor, in EDTA or Heparin stored at room temperature for not more than 48 h or at 4°C for not more than 4 days. Samples were kindly provided by Policlinico Ospedale Maggiore Ca’ Granda Milan, Italy, according to hospital ethical regulations (see Note 1).
2.3. Reagents and Chemicals

1. Purified water (e.g., Milli-Q, Millipore, MA, USA) (see Note 2).
2. 1× DPBS Dulbecco phosphate buffer saline (Euroclone S.p.A. Italy).
3. TURK or Trypan blue for cell count (Sigma).
5. Mounting Media plus DNA counterstain ready to use (Vectashield, Vector Laboratories, Inc. CA, USA).
6. Immersion oil, type FF (Cargille Laboratories, USA).
7. FISH Probes (CE, IVD), LSI D13S25 Spectrum Orange™, LSI 13q34 Spectrum Green™, CEP®12 Spectrum Orange™ (Abbott Molecular, Illinois, USA).

2.4. Reagents Preparation (See Note 3)

1. Red blood lysis buffer (RBL) (for red blood cell lysis): 0.15 M NH₄Cl, 9.93 mM KHCO₃, and 0.13 mM EDTA (ethylenediaminetetraacetic acid) in purified H₂O.
2. Carnoy’s fixative: mix 3 parts of methanol 100% and 1 part of Glacial acetic acid (Carlo Erba, Italy).
3. 2× SSC: mix 100 μl of 20× SSC (saline–sodium citrate buffer, Sigma) and 900 μl of purified H₂O.
4. Digestion buffer: mix 10 μl of 1 N HCl, 5 μl of 10% pepsin (Sigma, powder diluted in water at pH≤7.0), 989.5 μl of purified H₂O and bring it to 37°C (see Note 4).
5. Post fixative: mix 26 μl of 37% formaldehyde, 100 μl of 10× DPBS, 50 μl of MgCl₂ 1 M (powder diluted in water) in 824 μl of purified H₂O.
6. Denaturing solution: 70% Ultrapure formamide in 2× SSC: mix 700 μl of ultrapure formamide, 100 μl of 20× SSC (pH 5.3) and 200 μl of purified H₂O. Adjust pH to 7.0–8.0.
7. Ethanol solutions: dilute 100% ethanol in purified H₂O (700 μl in 300 μl for 70%, 850 μl in 150 μl for 85%).
8. Post-hybridization wash solution 1 (0.3% NP40 (Tergitol, Sigma) in 0.4× SSC): mix 155 ml of NP-40, 10 ml of 20× SSC in 488.5 ml of purified H₂O.
9. Post-hybridization wash solution 2 (0.1% NP40 in 2× SSC): mix 0.5 ml of NP-40, 50 ml of 20× SSC in 449.5 ml of purified H₂O.

2.5. Materials and Instruments

2. Glass Coverslips 18×18 mm (Zeuss super, Zeuss Padova, ITALY).
5. Cell count chamber (i.e., Burker chamber) (Brand GmbH, Germany).

2.6. Microscope Equipment and Filters

1. Microscope. For image acquisition and analysis, signals were evaluated using an Olympus BX61 microscope. A 100 W mercury lamp with a life maximum of about 200 h is recommended (see Note 5).
2. Camera. F-View II camera (Olympus, PA, USA).
3. Objectives. The objective has a profound influence on the brightness, resolution, and general quality of the image. A 40× objective in conjunction with 10× eyepieces is suitable both for scanning and for routine FISH analysis. When a higher magnification is needed, satisfactory results can be obtained with a 100× achromatic oil immersion objective (UPlanSApo 100× immersion objective, N.A. 1.40).
4. Immersion oil. Immersion oil used with immersion objectives should be formulated for low auto-fluorescence and specifically for fluorescence microscopy use (see Note 6).
5. Filters. Olympus U-MNIBA3 FITC for Spectrum Green probes, U-MWIG3 Cy3 for Spectrum Orange probes, U-MNUA2 DAPI.
6. Images were acquired using Cell^A software (Olympus) and elaborated with Adobe Photoshop 7.0 program (Adobe System Incorporated).

3. Methods

In this protocol we report the development of FISH starting from living not-fixed or fixed cells from standard cytogenetic pellet on microFIND® using specific probes for CLL, a common hemato-oncological disease, detecting chromosome 12 polisomy and the region of chromosome 13 13q14.3.

3.1. Device Assembly

1. microFIND® is operative once the microfluidic pad is attached to the coated side of the slide. For this purpose, the plastic package element hosting microfluidic pads (see Fig. 1a) acts as assembling tool.
2. The device is prepared by:
   (a) Holding the coated slide in your fingers from the short side and fitting it to the plastic element containing the microfluidic pad (Fig. 1a, b).
3.2. Cellular Sample Preparation

3.2.1. Cellular Suspension of Living Not-Fixed Cells from BM and PB

microFIND® is designed to perform FISH directly on cytogenetic pellet, prepared according to standard procedures, or on living not-fixed cells, prepared as a cellular suspension in isotonic buffer from patient blood. Below we report the protocol for the preparation of living not-fixed cells from BM and PB: cytogenetic pellet can be prepared with available standard protocols.

1. Store BM and PB patient samples either in EDTA or Heparin at room temperature or 4°C until usage (up to 4 days from collection if stored at 4°C).

2. Protocols below are designed to process volumes from 1 ml down to 0.1 ml (respectively “Standard” or “Reduced” volume of patient samples).

3. Standard volume of patient samples:
   (a) Place 0.5 ml of BM or 1 ml of PB in 15 ml centrifuge tube.
   (b) Add RBL to a volume of 10 ml.
   (c) Keep 5 min at 4°C.
   (d) Centrifuge at 425 rcf (relative centrifugal force) for 5 min.
   (e) Discard supernatant.
   (f) Resuspend again cells in 10 ml of RBL buffer.
   (g) Centrifuge at 600 rcf for 5 min.
   (h) Resuspend cells in 1 ml of 1× DPBS.
   (i) Transfer to 1.5 ml tube.
   (j) Wash twice in 1× DPBS by centrifuging in a microfuge at 500 rcf for 5 min.
   (k) Before loading in microFIND® resuspend cells in 1× DPBS at a concentration between 8,000 and 15,000 cells/μl (optimal concentration of cells suspension is 10,000 cells/μl), after cell count with a cell count chamber (e.g., Burker chamber).

4. Reduced volume of patient samples: For samples from PB a volume down to 0.5 ml can be processed following the same
procedure as above. For samples derived from BM a volume down to 0.1 ml can be processed with the following protocol:

(a) Place 0.1 ml of BM in 1.5 ml centrifuge tube.
(b) Add RBL to a volume of 1.5 ml.
(c) Keep 5 min at 4°C.
(d) Centrifuge at 425 rcf for 5 min in a microfuge.
(e) Discard supernatant.
(f) Resuspend again cells in 1.5 ml of RBL buffer.
(g) Centrifuge at 600 rcf for 5 min in a microfuge.
(h) Resuspend cells in 1 ml of 1× DPBS.
(i) Wash twice in 1× DPBS by centrifuging in microfuge at 500 rcf for 5 min.
(j) Before loading in microFIND® resuspend cells in 1× DPBS at a concentration between 8,000 and 15,000 cells/μl (optimal concentration of cell suspension is 10,000 cells/μl) (see Note 8).

1. Before use, pre-incubate microFIND® at 37°C (using a hot plate, or a slide hybridizer), for at least 2 min.

2. Keeping microFIND® on the hot plate/hybridizer, pipette 1.5 μl of cell suspension into the IN well and slightly aspirate from the OUT well on other side of the microchannel with the manual syringe or with the motorized syringe (at 25 ml/h) for a maximum of 2 s, to favor the loading of cell suspension into the microchannel (see Note 9) (Fig. 2).

3. Incubate for 4 min at 37°C.

4. Do not empty the microchannel: pipette 20 μl of fixative Carnoy in the bottom of the IN well with a 10 μl tip.

5. Wait for 2 min at 37 ± 1°C, then add another 30 μl of fixative into the IN well and aspirate completely from the OUT well.

6. Cells are now tightly immobilized in microFIND®.

7. Observe the microchannel area under a Bright Field microscope. Examine the number of nuclei per field under a 10× objective. A minimum of approximately 150 cells per field is recommended for optimum assay results (see Fig. 3 for an example of optimal cellular density inside the microchannel).

8. In case of a suboptimal cell concentration per field it is suggested to repeat the procedure of adhesion and fixation on a new microFIND® (see Note 10); moreover in case of cellular suspension with low cellular number, it is recommended to decrease the volume of the suspension buffer down to few μl (not more than 2–3 μl) and load 1.5 μl on microFIND® to recover most of the cells.
9. At this step microFIND® can be processed for FISH analysis or stored at room temperature in dry conditions, protected from light, for 24 h.

10. If patient cells must be reanalyzed it is recommended to prepare cytogenetic pellet from the remaining sample, keep it at −20°C, and perform the analysis on a new microFIND® following the protocol for standard cytogenetic pellets.

3.3.2. **Loading and Adhesion of Standard Cytogenetic Pellet**

1. Prepare a cytogenetic pellet according to the standard procedure.

2. Before cell loading in microFIND® it is recommended to evaluate cell density, to avoid cell clogging in the microchannel: after pellet preparation take 1 µl of cell suspension and spot on a standard glass slide; let the fixative evaporate and observe cell density under a Bright Field microscope at 10× magnification (see Fig. 4a as example of low cell density and Fig. 4b as optimal cell density).
3. If cell density is optimal, proceed with loading, otherwise dilute or concentrate the pellet with Carnoy fixative accordingly.

4. Load 1.5 μl of cytogenetic pellet into the IN well, at room temperature, and wait 2 min (see Note 11).

5. Put microFIND® at 37°C and wait until the complete evaporation of Carnoy’s fixative (~5–10 min) (see Note 12).

6. Cells are now tightly immobilized in microFIND®.

7. Observe the microchannel area under a Bright Field microscope. Examine the number of nuclei per field under 10× objective. A minimum of approximately 150 cells per field is recommended for optimum assay results (see Fig. 3).

8. If an optimum cell density has not been reached repeat steps 1 and 2 and check again by microscopy. This operation can be repeated several times until an optimal cell concentration is reached in the microchannel (see Note 13).

9. At this step microFIND® can be processed for FISH analysis or stored at room temperature in dry conditions, protected from light for 24 h.

3.4. FISH Protocol

After loading of microFIND® with cell suspension (either from living not-fixed cells or from standard cytogenetic pellet) all the reagents for FISH analysis (see Subheading 3.4.1 below) except the probe will be used according to the following scheme, from A to D (see Fig. 5).

1. Reagent loading (Fig. 5a): Dispense a drop of reagent in the IN well with a pipette (volume is indicated below).

2. Partial aspiration from the OUT well (Fig. 5b): to let the reagent flow inside the microchannel: aspirate with the manual syringe as shown in the figure (or with motorized syringe at 50 ml/h) until the reagent drop on the IN well is reduced to a minimum (see Fig. 5b).
3. Incubation (Fig. 5c): follow the indicated Subheading 3.4.1 for incubation conditions.

4. Removal of reagent excess (Fig. 5d) (see Note 14): aspirate the residual drop over the IN well, touching it with the suction cup of the syringe.

At the end of step D, if required according to the protocol, start again from step A with the next reagent.

FISH protocol consists of a series of reagent passes through the device as listed below. The hybridization with the fluorescent probe can be performed in two ways using the protocol for “Denaturation” or “Co-denaturation” (see Note 15). If using “Denaturation” for samples of living not-fixed cells, follow the complete protocol described below. For samples prepared from standard cytogenetic pellet start this protocol from step 5.

1. All the reagents must be used according to the above scheme (from A to D).
2. 50 μl of 2× SSC: 15 min at 37°C.
3. 50 μl of Digestion buffer: 10 min at 37°C.
4. 50 μl of 1× DPBS: 5 min at room temperature.
5. 50 μl of Post-fixative: 5 min at room temperature.
6. 50 μl of 1× DPBS: 5 min at room temperature.
7. 30 μl of EtOH 70%: 1 min at room temperature.
8. 30 μl of EtOH 85%: 1 min at room temperature.
9. 30 μl of EtOH 100%: 1 min at room temperature.
10. 50 μl of Denaturing solution: 3 min at 75°C (see Note 16).
11. 30 μl of EtOH 70%: 1 min at room temperature.
12. 30 μl of EtOH 85%: 1 min at room temperature.
13. 30 μl of EtOH 100%: 1 min at room temperature.

3.5. Probe Preparation

1. Most of commercial probes are provided already denatured, but if the probe is not denatured you will need to denature it separately following manufacturer instruction.
2. In the case of co-denaturation, the probe will be denatured with the sample DNA directly in the microchannel.
3. The amount of probe mix needed for each channel is 0.5 μl.

3.5.1. Hybridization with Denaturation (For Both Living or Fixed Cells)

1. After the last incubation with EtOH 100% (step 9, Subheading 3.4.1) empty the microchannel completely from the OUT well.
2. Place microFIND® at 60°C for 2 min to dry completely.
3. Load the probe by aspirating 0.5 μl of hybridization mix with a P2 pipette; to correctly fill the channel, direct the tip toward microchannel entry (see Fig. 6).
4. Wait a few seconds to let the probe fill the microchannel completely.

Fig. 6. Probe loading. Example of how to load the probe on the bottom of the “IN” well, by directing the tip toward the channel; the probe will fill the channel by capillarity.
5. Cover both wells with a drop (approximately 5–10 μl) of mineral oil to prevent evaporation of probe: use a 10 μl tip to pipette oil on the bottom of the IN well and on top of the OUT well.

6. Hybridize the samples on a slide hybridizer (ThermoBrite™ or similar) in controlled humidity conditions, however not above 75–80% relative humidity, following the probe manufacturer’s suggested time and temperature. If you don’t have a slide hybridizer, you can reach these conditions incubating microFIND® in a closed box with a vessel filled with saturated NaCl solution on the bottom, in a dry incubator (Note 17).

3.5.2. Hybridization with Co-denaturation

Co-denaturation is suitable and reproducible for CEP (Chromosome Enumeration Probes) LSI (Locus Specific Identifier) probes although some times with lower signal to noise ratio.

For Samples Derived from Living Not-Fixed Cells

1. After the first ethanol scale (step 9, Subheading 3.4.1), empty the microchannel completely from the OUT well.
2. Place microFIND® at 60°C for 2 min to dry completely.
3. Load the probe, pre-heated at 75°C, by aspirating 0.5 μl of hybridization mix using a P2 pipette: to correctly fill the channel direct the tip toward microchannel entry (see Fig. 6).
4. Wait a few seconds to let the probe fill the microchannel completely.
5. Cover both wells with a drop (approximately 5–10 μl) of mineral oil to prevent evaporation of probe.
6. Co-denature the samples at 75°C for maximum 5 min.
7. Hybridize the samples following probe manufacturer’s suggested time and temperature.

For Standard Cytogenetic Pellet

After the adhesion procedure (Subheading 3.3.2) leave microFIND® at 37°C for at least 5 min to ensure complete fixative evaporation, then follow the previous Subheading “For Samples Derived from Living Not-Fixed Cells” starting from step 3 (Load the probe), then proceed with co-denaturation, by directly loading the probe in microFIND®, as described above, incubating at 75°C for maximum 1.5 min, then hybridizing the samples following probe manufacturer’s suggested time and temperature.

3.6. Post-hybridization Washes

1. After hybridization, remove the pad grabbing it by its flap and pulling it away from the slide (Fig. 7).
2. Perform post-hybridization washes by dipping the slide in coplin jars containing: Wash Solution 1 (pre-heated at 73°C) for 30 s and Wash Solution 2 at room temperature for 30 s.
3. Air dry the slide and mount coverslip with 4 μl of Vectashield per microchannel; the slide is then ready for the analysis.
1. Slides are analyzed by fluorescence microscopy.

2. Cells that are suitable for analysis and evaluation are located in the microchannel area (see Fig. 8a): do not analyze cells that are located in the correspondence of the IN and OUT wells if they appear as aggregates.

3. First the sample is analyzed at low magnifications (10×) to identify, through DAPI inspection, cells of interest (see Fig. 8b for a selected area of analysis).

4. A detailed evaluation of 200 cells at high magnification (100×) (scored per probe/per patient) showed that an average of 97.8% of cells were clearly positive for both probes.

5. Examples of FISH experiments on four CLL patients are reported in Fig. 9 (100× magnification). Three patients resulted
positive and one negative for the genetic lesions tested; white arrows in Patient (Pat.) 1, 2, 3 indicate the presence of a specific lesion in the nucleus.

6. The percentage of positivity for each single lesion is reported in Table 1.
1. Patient samples consist of white blood cells isolated from total blood and resuspended in saline buffer. Different methods of purification are suitable, such as density gradient centrifugation (Ficoll method), hypotonic lysis buffer, or RBL buffer treatment, as described in this protocol.

2. Purified H₂O must have a controlled pH of 7.0. Uncontrolled pH in the water source can lead to degradation of fluorophores during post-hybridization washes.

3. It is recommended to prepare fresh solutions each time.

4. Prepare digestion buffer 15 min before use and keep at 37°C until use. It has been tested that Pepsin reaches optimal membrane digestion activity after a pre-heating time.

5. Routine microscope cleaning and periodic preventive maintenance by the manufacturer’s technical representative are recommended; check the usage time of the microscopy mercury lamp: old lamps can result in a worse signal/noise ratio and therefore can produce inappropriate results. Use the indicated filters according to the information on probe’s fluorophores; signal brightness can be improved using the correct filters.

6. Immersion oil contributes to two characteristics of the image viewed through the microscope: finer resolution and brightness. These features are most critical under high magnification: therefore use an immersion oil with refractive index suitable to your objective and matching the mounting media employed.

7. The polymeric material of the microfluidic pad slowly releases a minimal amount of volatile compounds that could modify the properties of the slide coating. This is the reason why slides and pads have separated packages. For this reason, the assem-

### Table 1

**Percentages of positivity of specific probes, with respect to the observed genetic aberrations**

<table>
<thead>
<tr>
<th>N=Negative</th>
<th>P=Positive</th>
<th>+12</th>
<th>del 13q14.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pat. 1</td>
<td>P</td>
<td>63%</td>
<td>N</td>
</tr>
<tr>
<td>Pat. 2</td>
<td>P</td>
<td>53.5%</td>
<td>P</td>
</tr>
<tr>
<td>Pat. 3</td>
<td>N</td>
<td>100%</td>
<td>P</td>
</tr>
<tr>
<td>Pat. 4</td>
<td>N</td>
<td>99%</td>
<td>N</td>
</tr>
</tbody>
</table>

+12 refers to trisomy of chromosome 12, del 13q14.3 to deletion of the specific region of chromosome 13

### 4. Notes

1. Patient samples consist of white blood cells isolated from total blood and resuspended in saline buffer. Different methods of purification are suitable, such as density gradient centrifugation (Ficoll method), hypotonic lysis buffer, or RBL buffer treatment, as described in this protocol.

2. Purified H₂O must have a controlled pH of 7.0. Uncontrolled pH in the water source can lead to degradation of fluorophores during post-hybridization washes.

3. It is recommended to prepare fresh solutions each time.

4. Prepare digestion buffer 15 min before use and keep at 37°C until use. It has been tested that Pepsin reaches optimal membrane digestion activity after a pre-heating time.

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6. Immersion oil contributes to two characteristics of the image viewed through the microscope: finer resolution and brightness. These features are most critical under high magnification: therefore use an immersion oil with refractive index suitable to your objective and matching the mounting media employed.

7. The polymeric material of the microfluidic pad slowly releases a minimal amount of volatile compounds that could modify the properties of the slide coating. This is the reason why slides and pads have separated packages. For this reason, the assem-
bling of microFIND® must be done when ready for the FISH protocol; the device must therefore be prepared and used within 1 h. During these operations, avoid touching the slide coating since this would result in degraded performance of the device.

8. Samples for FISH can also be derived by procedure of cell purification, like IMAC procedure (Immuno-affinity metal assisted chromatography) or FACS (fluorescence activated cell sorting); for instance, this is the case for the analysis of plasma cells deriving from multiple myeloma patients. In such case the amount of selected cells is often low and the preparation of a cytogenetic pellet for standard FISH can cause dramatic cellular loss: therefore cellular suspension can be prepared directly from living cells resuspended in a small volume of isotonic buffer. It is suggested to reduce the volume in not more than 5 μl of isotonic buffer and directly load the sample on the device to be able to recover all the cells present in the sample. Cell fixation and FISH analysis will occur inside microFIND®, avoiding cellular loss.

9. After loading the cell suspension of living not-fixed cells, do not completely aspirate the liquid from the microchannel. This would dramatically impair cell adhesion. It is important that no air bubbles pass through the channel before the Carnoy fixative has been loaded.

10. microFIND® is able to efficiently immobilize living not-fixed cells in isotonic buffer with a low amount of protein: salt and water-based buffers inactivate the surface after the first incubation with the cellular suspension; therefore it is not recommended to reload the cellular suspension after the first incubation and cell immobilization.

11. For an effective adhesion, be sure that your sample has been resuspended in fresh Carnoy fixative. Perform a check for cell density (see Fig. 4): if it is too concentrated proceed with a dilution of the pellet with fresh Carnoy, otherwise if too diluted, centrifuge the pellet again and resuspend in a lower volume. Repeat the check for cell density; a proper cellular dilution is crucial for an optimal result; refer to Fig. 4b as a correct reference.

12. Carnoy’s fixative evaporation time depends on environmental factors like room temperature and relative humidity mainly. Evaporation will take a longer time in a highly humid environment, and vice versa.

13. Contrary to the living cell preparation, cytogenetic pellet can be repeatedly loaded in microFIND® to reach an optimal cellular number and distribution, particularly if the sample has a low cell density. In case of multiple loading, be sure that the
cytogenetic pellet is a uniform suspension, free of clumps. This is a critical point since cell aggregation will impair the correct loading of microFIND®. Between subsequent loading Carnoy fixative must be completely evaporated. It is possible to speed up this step by placing microFIND® at 75°C for few minutes.

14. To eliminate the liquid in excess on the IN well it is sufficient to place the suction cup over the residual volume while aspirating and the drop should disappear in an instant. Be sure that during all the incubations the reagent reservoir on the IN well DOES NOT DRY OUT: in dry environmental conditions (relative humidity below 20%) check the volume of the drop and, if it decreases rapidly, add an equal amount of reagent volume.

15. Recently the protocol of probe co-denaturation has been proposed as alternative to significantly reduce the protocol duration. However, using co-denaturation with microFIND®, reproducible results have been obtained only with CEP probes, while with LSI probes the results have not always been satisfactory, suggesting that the denaturation protocol must be recommended as first choice.

16. Check the heating block temperature before starting probe denaturation.

17. Do not incubate microFIND® in incubators for cell culture: the high humidity can cause condensation on the slide and consequent detachment of the polymeric microfluidic pad, leading to probe diffusion and incorrect hybridization conditions.

Acknowledgments

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References