

# Chapter 2

## Biomarkers of Cell Proliferation in Carcinomas: Detection of Angiogenesis and Infiltrated Leukocytes

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### Summary

Angiogenesis is an important marker for tumor growth, development, and metastasis. There are many studies to detect angiogenesis, for instance by microvessel density (MVD), though several of the studies to MVD measurement show opposite results. Measurement of MVD is a nontime-related measurement, whereas angiogenesis is a dynamic process; therefore, measurement of proliferating endothelial cells is thought to be a better method. We have shown in studies that measurement of active proliferating endothelial cells by double staining is a better marker, compared to MVD measurement. Next to angiogenesis, leukocyte infiltration in a cancer has a prognostic value. A large infiltration of leukocytes in a tumor correlates with a better survival. It is known that the correlation between leukocyte infiltration and angiogenesis is marked by adhesion molecule expression on endothelial cells. In vitro experiments show that active proliferating endothelial cells downregulate adhesion molecule expression on the cell membrane. It is generally assumed that this results in vivo in an inhibition of leukocyte infiltration in this specific area. Because immunohistochemical techniques cannot detect exact amounts of adhesion molecules in physiological environments this interaction has not been demonstrated. This chapter shows a technique based on flowcytometry by which these analyses can be performed. In short a tissue part is dissolved in a single-cell suspension, stained for specific characteristics and measured by FACS analysis. In this chapter we will show several techniques to detect proliferating endothelial cells in a tissue.

**Key words:** Tumor; Angiogenesis; Leukocytes; Adhesion molecule expression; Flowcytometry.

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### 1. Introduction

Prognostic factors such as Tumor, Node, Metastasis (TNM) stage predict cancer survival. This staging shows an indication to survival, but is not sufficient to show accurate survival. Several other parameters have been proposed to improve prognostic

criteria, among which are angiogenic potential and infiltration by inflammatory cells (1–3). These two phenomena are interrelated; therefore, studies to assess the value of both phenomena for the prognosis have been made in several carcinomas. Angiogenesis is essential for tumor growth and metastasis, and is regulated by tumor cells through the production of proangiogenic factors such as vascular endothelial cell growth factors (VEGFs) and fibroblast growth factors (FGFs), and angiostatic factors such as platelet factor-4, thrombospondin-1, angiostatin, and endostatin (4, 5). Detection of single or combination of these factors in tissue did not accurately show angiogenic activity and survival. Markers for endothelial cells as CD31, CD34, CD105, and Von Willebrand factor show small nonsignificant differences. Different angiogenesis measurement techniques as microvessel density, image analysis software, growth-factor detection magnetic resonance imaging, and positron emission tomography are used (6, 7). Measurement of angiogenesis is commonly performed by the assessment of microvessel density (MVD) (8). This parameter is controversial since it might not be a reliable indicator of ongoing angiogenesis. It has been suggested that angiogenesis is best evaluated through detection of proliferating ECs (9).

In contrast to angiogenesis the amount of leukocytes is assumed to be important for cancer regression, in a way that high amounts of several different leukocyte subsets show a better prognosis (1). Therefore leukocyte infiltration in a carcinoma is an important prognostic factor. Though it is not known which subset exactly is important for the regression of a cancer, it is suggested that different cancers react different to several leukocyte subsets.

Communication between resident tissue cells and circulating leukocytes is mediated by the endothelial cell layer. Resident antigen-presenting cells release cytokines like TNF- $\alpha$ , IL-1, IFN- $\alpha$ , and histamine in response to an inflammatory reaction (10). Endothelial cells respond to the cytokine release by expressing adhesion molecules on their surface in order to enable leukocytes to adhere to their surface and infiltrate the surrounding area. Not only in inflammatory tissue is leukocyte infiltration important. Leukocyte infiltration in cancer tissue is thought to be a prognostic factor in a way that tumors with high leukocyte counts have a better prognosis (11). Local release of proinflammatory cytokines induces biosynthesis and luminal expression of vascular adhesion molecules as ICAM-1, VCAM, and E-selectin. E-selectin is known to be of importance in the rolling of leukocytes in contrast to VCAM and ICAM which have been shown to be important in firm leukocyte endothelial cell adhesion (12).

We are able to determine adhesion molecule expression on endothelial cells with our developed flowcytometric method. Using a double staining, on a single-cell suspension, with endothelial

markers and adhesion molecules we can easily detect relative expression of adhesion molecules on endothelial cells.

Next to detection of proliferating ECs, we will show in this chapter the relationship of proliferating EC with adhesion molecule expression on its membrane and the relationship with leukocyte infiltration.

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## 2. Materials

### 2.1. Immunohistochemistry Needs

1. Materials:
  - (a) Object slides
  - (b) Cutter for 4- or 5- $\mu$ m slides
  - (c) Pipette
  - (d) Mount slides
  - (e) Microscope
  - (f) Incubation room
2. Tissues: paraffin, fresh or frozen consecutive sections cut at 4 or 5  $\mu$ m.
3. Solutions (*see Note 1*):
  - (a) Deparaffinize: xylene  
(fresh from stock)
  - (b) Alcohol concentrations: 100%, 96%, 70%  
(fresh from stock)
  - (c) PBS 0.01 M (PBS):  
NaCl 218.8 g  
 $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  35.4 g Dilute in 5 l distilled water  
 $\text{KH}_2\text{PO}_4$  5.38 g  
Store at 5°C, for 2–4 months  
Needs 5 $\times$  dilution before use control; for PH: 7.2–7.4  
(conductivity at 25°C: 15.7 ms)
  - (d) 5% BSA/PBS:  
5 g of BSA (Sigma, St Louis, MO) in 100 ml PBS  
Store at –20°C, for 6–12 months
  - (e) 0.5%BSA/PBS:  
0.5 g of BSA (Sigma, St Louis, MO) in 100 ml PBS  
or  
1:10 dilution of 5%PBS/BSA in PBS.  
Store at –20°C, for 6–12 months

- (f) Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0):

Tri-sodium citrate (dihydrate) 2.94g. Dilute in 1 l distilled water

Adjust pH to 6.0 with 1 N HCl

Store at 5°C, for 6–12 months

- (g) DAB:

1 ml pure DAB + 9 ml Tris-HCl (0.05 M; pH 7.6) + 10  $\mu$ l  $H_2O_2$

Store at -20°C, for 6–12 months

## 2.2. Flowcytometry Needs

### 1. Materials

- (a) Flowcytometer (FACSCaliber)
- (b) Cryocutter
- (c) Warm water bath
- (d) Vortex
- (e) Centrifuge
- (f) Vacuum
- (g) Pipette
- (h) Ice
- (i) Microscope, cell-counter glass
- (j) Filter, 50  $\mu$ m
- (k) FACS tubes
- (l) FACS tube holders
- (m) Eppendorf tubes

2. *Tissues*. Cut tissues, in cryo, in 30- $\mu$ m sections and put them in a frozen 10-ml tube and put them on dry ice (*see Note 2*). When not directly used store tissue sections in a fridge at -20°C (maximum 12 months).

### 3. Solutions:

- (a) Collagenase:
- (b) Collagenase 5 mg/ml (50mm of collagenase in 10ml Bidest)
- (c) Store at -20°C, for 6–12 months
- (d) Dispase:
- (e) Dispase 1 mg/ml (10mg Dispase in 10ml Bidest)  
Store at -20°C, for 6–12 months
- (f) *Paraformaldehyde 0.1%*. 1% Paraformaldehyde (10 g of Paraformaldehyde diluted in 100 ml bidistilled water, pH set to 7.3 with NaOH) Store at -20°C, for 6–12 months  
Before use dilute paraformaldehyde till 1% with PBS (0.01 M) 1:10

### 3. Methods

For the immunohistochemical methods paraffin, fresh as well as fresh frozen tissues, can be used. The protocol we show is made for paraffin-embedded tissues. When fresh or frozen tissues are used use an extra step of incubation with 1% paraformaldehyde (follow protocol flowcytometry) is needed instead of the deparaffinization steps. This protocol can be followed afterward.

For all fresh frozen tissues, best results are obtained when the tissues are directly fixated when obtained; proliferation of cells is dependent on nutrients. Next to proliferation the amount of adhesion molecule expression will also decrease in time if the tissue is not fixated (*see Note 2*).

The following protocols will guide you through a number of techniques to detect proliferating EC, leukocytes, and adhesion molecule expression on EC.

#### **3.1. Ongoing Angiogenesis Measured by Immunohistochemistry**

##### *3.1.1. Protocol: Ongoing Angiogenesis Measured by Immunohistochemistry*

The classical way to detect angiogenesis by MVD detection has been shown to register a static situation of the number of vessels in a tissue. We and others have shown that the number of active proliferating EC is a better method to detect ongoing angiogenesis. This protocol shows how a double staining of Ki67 (proliferation marker) with EC marker cocktail (CD31/CD34) can be performed.

1. For this research use adjacent sections, because after staining the tumor sites might be difficult to find, it is useful to stain one of the adjacent sections with hematoxylin/eosin. This gives a better result than staining the section with hematoxylin/eosin, because the blue staining might be difficult to see after counterstaining with hematoxylin.
2. Deparaffinize (2× xylene for 5 min)
3. Rehydrate (2× 100% alcohol; 1× Alcohol 96%; 1× alcohol 70%; (each 5 min)
4. Twenty minutes in methanol + 3% H<sub>2</sub>O<sub>2</sub> (to eliminate endogen peroxidase)
5. Rinse with H<sub>2</sub>O
6. Citrate-buffer pH 6; incubate at boiling temperature for 10 min. Permeabilization of the cell membrane is important for Ki-67 staining in the nucleus.
7. Thirty minutes cooling down
8. 3 × wash with PBS
9. Dry slides
10. Five percent BSA in PBS; 100 µl per slide, use a humid environment for 30 min. Blocks a nonspecific binding

11. Clean slides by putting them in an oblique position
12. Add First antibody Ki-67 (Rabbit anti-Human; NeoMarker, Freemont, CA.) 100  $\mu$ l per slide, incubate for 60 min in a humid environment.  
1:200 dilution  $\Rightarrow$  10  $\mu$ l in 2 ml 0.5% BSA in PBS (*see* **Notes 3 and 4**)
13. 3 $\times$  wash with PBS
14. Add complementary  $\alpha$ -rabbit<sup>b</sup> (Swine-anti-rabbit<sup>biotinc</sup>; DAKO, Glostrup, Denmark),  
100  $\mu$ l per slide, incubate for 30 min in a humid environment  
1:200 dilution  $\Rightarrow$  5  $\mu$ l + 1 ml 0.5% BSA in PBS 0.5
15. 3 $\times$  wash with PBS
16. Avidine-Bovine-Complex HRP (ABC-complex HRP; DAKO, Glostrup, Denmark), firm protocol, 100  $\mu$ l per slide, incubate for 30 min in a humid environment
17. 3  $\times$  wash with PBS
18. DAB solution (DAB, Sigma, Zwijndrecht, the Netherlands)  
 $\Rightarrow$  *Work with gloves (DAB is carcinogenic)*  
1 ml pure DAB + 9 ml Tris-HCl (0.05 M; pH 7.6) + 10  $\mu$ l H<sub>2</sub>O<sub>2</sub>: DAB is photosensitive, use dark environment, use 100  $\mu$ l per slide, control staining under microscope, when clear specific staining appears stop the reaction by adding H<sub>2</sub>O
19. 2 $\times$  wash with PBS
20. Antibody cocktail CD31 + CD34 (both mouse-antihuman; DAKO(CD31)/QBEND-10, novocastra, Uden, The Netherlands(CD34)), 100  $\mu$ l per slide, incubate for 60 min in a humid environment  
1:100/1:50 dilution (CD31/CD34) 10  $\mu$ l + 20  $\mu$ l + 1 ml
21. 3 $\times$  wash with PBS
22. Add complementary  $\alpha$ -mouse<sup>b</sup> (anti-rabbit<sup>biotinc</sup>), 100  $\mu$ l per slide, incubate for 30 min in a humid environment  
1:200 dilution  $\Rightarrow$  5  $\mu$ l + 1 ml 0.5% BSA in PBS 0.5
23. 3 $\times$  wash with PBS
24. Avidine-Bovine-Complex AP (ABC-complex AP; DAKO), company's protocol, 100  $\mu$ l per slide, incubate for 30 min in a humid environment
25. 3  $\times$  wash with PBS
26. Alkaline phosphatase substrate KIT III Blue (Vector Laboratories, Inc., Burlingame, CA); photosensitive, use dark environment, use 100  $\mu$ l per slide, control staining under

microscope, when nice specific staining stop the reaction by adding  $H_2O$  use Tris 0.8 M pH 8.2 dilute the alkalic phosphatase (AP) in

27. Add a film of insol mount to protect AP from alcohol
28. Let slides air dry completely
29. Dehydrate and mount slides

### **3.2. Immune System (Leukocyte Subsets)**

The presence of immune cells in tumors is generally seen as a favorable prognostic parameter. This is evident for leukocyte subsets, including T-lymphocytes, PMNs, and macrophages (13, 14). However, for tumor-associated macrophages adverse effects have been described (15, 16). Tumor infiltration by leukocytes is regulated by a number of inflammatory cytokines, the combination of which presumably determines the amount and composition of the infiltrate. In addition, infiltration is also affected by regulators of angiogenesis. Both inhibitory and stimulatory effects were found for VEGF and other angiogenic growth factors during angiogenesis (17–19). Angiogenesis is inversely correlated with leukocyte infiltration in a way that leukocyte infiltration is lower when high angiogenic activity is measured in a tissue (also cancer tissue). Therefore leukocyte infiltration is indicative of endothelial cell proliferation level.

#### **3.2.1. Protocol: Immuno- histochemical Staining of the Immune System (Leukocyte Subsets)**

1. Deparaffin (2× xylene for 5 min)
2. Rehydrate (2× 100% alcohol; 1× Alcohol 96%; 1× alcohol 70%; (each 5 min) )
3. Twenty minutes in methanol + 3%  $H_2O_2$  (to eliminate endogen peroxidase)
4. Rinse with  $H_2O$
5. Citrate buffer pH 6; incubate at boiling temperature for 10 min
6. Thirty minutes cooling down
7. 3× wash with PBS
8. Dry slides
9. Five percent BSA/PBS 100µl per slide, use a humid environment for 30 min
10. Clean slides by putting them in an oblique position
11. Add First antibody (all mouse-antihuman):

CD3 (DAKO)	1:50 dilution
CD8 (Novocastra, Valkenswaard, the Netherlands)	1:100 dilution
CD16 (NeoMarker, Freemont, CA.)	1:50 dilution
CD20 (DAKO)	1:1000 dilution
CD68 (DAKO)	1:100 dilution

Hundred microliters per slide, incubate for 60 min in a humid environment.

All dilutions are made with 0.5% BSA in PBS

12. 3 × wash with PBS
13. Add complementary  $\alpha$ -mouse<sup>b</sup> IG (rabbit-antimouse<sup>biotine</sup>; DAKO, Glostrup, Denmark), 100  $\mu$ l per slide, incubate for 30 min in a humid environment  
1:200 dilution  $\Rightarrow$  5  $\mu$ l + 1 ml 0.5% BSA in PBS 0.5
14. 3 × wash with PBS
15. Avidine-Bovine-Complex HRP (ABC-complex HRP; DAKO, Glostrup, Denmark), firm protocol, 100  $\mu$ l per slide, incubate for 30 min in a humid environment
16. 3 × wash with PBS
17. DAB-solution (DAB, Sigma, Zwijndrecht, the Netherlands)  
 $\Rightarrow$  *work with gloves (DAB is carcinogenic)*  
1 ml pure DAB + 9 ml Tris-HCl (0.05 M; pH 7.6) + 10  $\mu$ l  $H_2O_2$ ; DAB is photosensitive, use dark environment, use 100  $\mu$ l per slide, control staining under microscope, when nice specific staining stop the reaction by adding  $H_2O$
18. 2 × wash with PBS
19. Counterstain with hematoxinilin
20. Dehydrate (1 × 70% ethanol, 1 × 96% ethanol, 2 × 100% ethanol (each for 5 min))
21. Mount slides

### **3.3. Adhesion Molecule Expression on EC**

Detection of amount or percentage of endothelial cells (EC) (not blood vessels) and adhesion molecules on EC is not possible with immunohistochemical methods. It is plausible that each EC has the capacity to express adhesion molecules on its surface. Therefore single-cell suspensions are needed to detect the percentage of EC in a tissue. We developed a technique based on flowcytometric detection of endothelial cells in a tissue solution. With this technique an accurate percentage of endothelial cells in a tissue can be detected. For this technique paraffin-embedded, fresh frozen as well as fresh tissue can be used. Thirty-micrometer tissue sections are initially dissolved to form a single-cell suspension. This suspension can be stained for any set of specific markers; therefore, this technique is not solely limited to endothelial cell detection, but is a general method to detect leukocytes, tumor cells, etc. This technique has a very high reproducibility no interobserver variation, and is easy to perform. For this protocol we used frozen tissues, because a number of adhesion molecules are not detectable after paraffin embedding. When paraffin-embedded tissues are used in other



setting use **steps 1 and 2** shown in immunohistochemical protocol instead of paraformaldehyde fixation.

The protocol shows three sections (**Subheadings 3.3.1–3.3.3**) to guide a reader through the entire process.

*3.3.1. Protocol: Standard  
Preparation of Single  
Cells for Flowcytometric  
Analysis*

Safety: Be careful with tumor samples and even with stained cells!

1. The tissue sections of 30  $\mu\text{m}$  are cut in a cryocutter. The tissues can be put directly in a 10-ml tube; if not used immediately, the tissues can be stored at  $-20^{\circ}\text{C}$ .
2. All materials and solutions need to be prepared before starting the protocol.
3. Tissues in a 10-ml tube need fixation, a 1/2 or 1 ml paraformaldehyde 1% is added and incubated for 60 min
4. After 1 h use centrifuge (RZB = 689) for 5 min.

Then take the fluid away using the vacuum

5. Single-cell suspension preparation:

Collagenase 5 mg/ml + Dispase 1 mg/ml (ten collagenase: one dispase) (*see Notes 5 and 6*)

Add 1 ml collagenase/dispase solution to every tube

Put tubes for 15 min in a water bath  $37^{\circ}\text{C}$

Use a 1,000- $\mu\text{l}$  pipette to triturate the liquid with tissue parts.

Repetitive pipetting will result in a single-cell suspension.

Make a single-cell suspension (if this does not work out for the tissue a brief titration in time and concentration of collagenase and dispase must be performed)

6. After 15 min, put the tubes directly on ice and use 9 ml PBS to fill the tube (this stops the enzyme reaction)
7. Take cell counter and control the single-cell suspension (all single cells)
8. Use centrifuge (RZB = 398) for 5 min.

*Remove the fluid using the vacuum, be careful not to lose cells in each vacuum step!!* Repeat the rinsing step twice

9. Divide the single-cell suspension over FACS tubes:

For each 10-ml tube (after vacuum):

Add  $X$  times 200  $\mu\text{l}$  0.5% BSA in PBS, where

$X$  is total number of FACS tubes for one tissue sample needed for the experiment

Suspend the fluid using the vortex and divide it in the  $X$  FACS tubes

10. Use centrifuge (RZB = 398) for 5 min.

Then take the fluid away using the vacuum.  
*From this step on perform all experiments on ice!!!*

*3.3.2. Protocol: Detection  
 of Endothelial Cells in  
 Single-Cell Suspension*

1. For every step a control sample must be used.
2. Make primary antigen solution  $\Rightarrow$  CD-31 (mouse-antihuman; DAKO)  
 1:100 dilution in 0.5% BSA in PBS. Use 20  $\mu$ l solution per FACS tube. Shake tubes (vortex) to suspend the single cells in the solution. Incubate for 1 h (on ice)
3. After 1 h add 200  $\mu$ l, 0.5% BSA in PBS per FACS tube  
 Use centrifuge RZB = 398 for 5 min.  
 Then take the fluid away using the vacuum.  
 Repeat rinsing two more times
4. Make secondary antigen solution  $\Rightarrow$  biotin-conjugated rabbit-antimouse (DAKO)  
 1:50 dilution in 0.5% BSA in PBS  
 20  $\mu$ l solution/FACS-tube. Shake tubes (vortex) to suspend the single cells in the solution  
 Incubate for 1 h (on ice)
5. After 1 h add 200  $\mu$ l 0.5% BSA in PBS per FACS tube  
 Use centrifuge RZB = 398 for 5 min.  
 Then take the fluid away using the vacuum.  
 Repeat rinsing two more times
6. Make tertiary antigen solution  $\Rightarrow$  phycoerythrin (PE)-conjugated streptavidin (10  $\mu$ g/ml; DAKO) 1:25 dilution in 0.5% BSA in PBS  
 Twenty microliters solution/FACS tube. Shake tubes (vortex) to suspend the single cells in the solution  
 Let antigens incubate for 1/2 h (on ice and cover because coloring is light sensitive)
7. After 1 h put 200  $\mu$ l 0.5% BSA in PBS/FACS tube  
 Use centrifuge RZB = 398 for 5 min  
 Then take the fluid away using the vacuum  
 Repeat rinsing two more times
8. The cells are now ready for FACS analysis

*3.3.3. Protocol: Adhesion  
 Molecule Expression on  
 Endothelial Cells*

For double staining of adhesion molecules on the surface of endothelial cells, **step 18** should be skipped and the following steps need to be performed (*see* **Notes 7–9**):

1. Direct FITC-conjugated antibodies are needed  
 ICAM-1 1:50 dilution in 0.5% BSA in PBS + 5% normal mouse serum

VCAM-1 1:50 dilution in 0.5% BSA in PBS + 5% normal mouse serum

E-selectin 1:40 dilution in 0.5% BSA in PBS + 5% normal mouse serum

The presence of 5% normal mouse serum is used to prevent aspecific binding to the primary staining.

20  $\mu$  solution/FACS tube. Shake tubes (vortex) to suspend the single cells in the solution

Let antigens incubate for 2 h (on ice and cover because coloring is light sensitive)

2. After 2 h put 200  $\mu$ l 0.5% BSA in PBS/FACS tube

Use centrifuge RZB = 398 for 5 min.

Then take the fluid away using the vacuum.

Repeat rinsing two more times

3. The cells are now ready for FACS analysis (**Fig. 1**) (*see Note 10*).

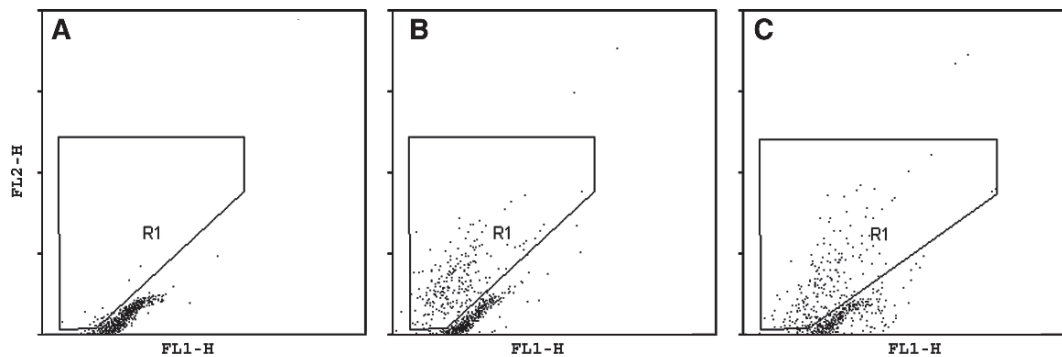


Fig. 1. Example of flowcytometric results. (A) is a control. The gate plotted shows only 0.4% of hits. In the CD31-stained solution (B) this number of hits (CD31 positive) is 11.35%. This means 11% of the total amount of cells in this tissue (colorectal cancer) is endothelial cell. Furthermore, the x-value in the plotted gate is 6.5; this means that the standard autoimmunefluorescence, for FL1-H, of the endothelial cells is 6.5. This value of 6.5 can be set to zero when compensation with software delivered with a flowcytometer is used. In (C) the amount of adhesion molecule ICAM-1 on endothelial cells is plotted. What can be seen is that all positive ICAM-1 cells move to the right, not only endothelial cells. The x-value in the gate is 10.3; this is the parameter for the amount of ICAM-1 positivity of the endothelial cells. There is an equal amount of cells stained within the gate (11.30%, all endothelial cells) which means no loss of endothelial cells in the negative cloud right of the gate. In some cases this cloud fully disappears in the endothelial negative cloud; therefore, compensation with software can be used.

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## 4. Notes

1. All solutions are prepared with distilled water, unless stated otherwise.
2. Consecutive tissues are essential in immunohistochemistry to identify tumor regions in HE-stained tissues and be marked on the double-stained (Ki67-CD31/34) slides.
3. Antibodies from different stocks or different commercial sources might need other dilution.
4. All antibodies collagenase and dispase dilutions can be prepared, aliquoted, and stored in a freezer at  $-20^{\circ}\text{C}$  to ensure consistent conditions.
5. Repeated vigorous pipetting with a 1-ml pipette during collagenase and dispase treatment results in a single-cell suspension. The use of a mesh is also possible, though some tissue can get retained by the mesh and this might influence the results.
6. Tissues should be handled carefully particularly in the fresh frozen tissues. When cells are damaged or too many cell clots still exist, the FACS analysis will not accurately show percentages. Different tissues might need different concentration of collagenase/dispase solution and different incubation time of collagenase/dispase at  $37^{\circ}\text{C}$  waterbath. A microscopic evaluation of the single-cell conditions can be performed after preparation of the single-cell suspension. Propidium iodide ( $20\mu\text{g}/\text{ml}$  in PBS, permeabilization in 70% ethanol) can be used as a control for intact cells.
7. Before flow-cytometric measurement the cells need to be suspended with a vortex resulting in an adequate single-cell suspension.
8. Be aware that the fluorescent particles are light sensitive; therefore, covering of the samples, after admitting, is necessary.
9. Quantification with flow cytometry (in our study a FACSCaliber), with at least two detection lasers (FITC (525 nm) and APC (660 nm)) was used. The use of compensation rate for red or green light can be helpful.
10. This technique can be adapted for many strategies to identify different cell types in a tissue. The basic procedure, preparation of a single-cell suspension, can be used in almost all tissues.

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## Acknowledgments

The author would like to thank the Department of Pathology, University Hospital Maastricht, for support and advice.

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Inflammation and Cancer

Methods and Protocols: Volume 1, Experimental Models  
and Practical Approaches

Kozlov, S.V. (Ed.)

2009, XIV, 378 p. 75 illus., Hardcover

ISBN: 978-1-934115-14-5

A product of Humana Press