

## ibidi Application Guide

# Immunofluorescence Assays

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### Selected Publications

C. Xu, et al. *NPTX2 promotes colorectal cancer growth and liver metastasis by the activation of the canonical Wnt/beta-catenin pathway via FZD6*. *Cell Death & Disease*, 2019, 10.1038/s41419-019-1467-7

[read abstract](#)

Kobayashi, T., et al. *Principles of early human development and germ cell program from conserved model systems*. *Nature*, 2017, 10.1038/nature22812

[read abstract](#)

H. Tada et al. *Porphyromonas gingivalis Gingipain-Dependently Enhances IL-33 Production in Human Gingival Epithelial Cells*. *PloS one*, 2016, 10.1371/journal.pone.0152794

[read abstract](#)

N. J. Foy, M. Akhrymuk, A. V. Shustov, E. I. Frolova and I. Frolov. *Hypervariable Domain of Nonstructural Protein nsP3 of Venezuelan Equine Encephalitis Virus Determines Cell-Specific Mode of Virus Replication*. *Journal of Virology*, 2013, 10.1128/jvi.00720-13

[read abstract](#)

# The Principle of Immunofluorescence Assays

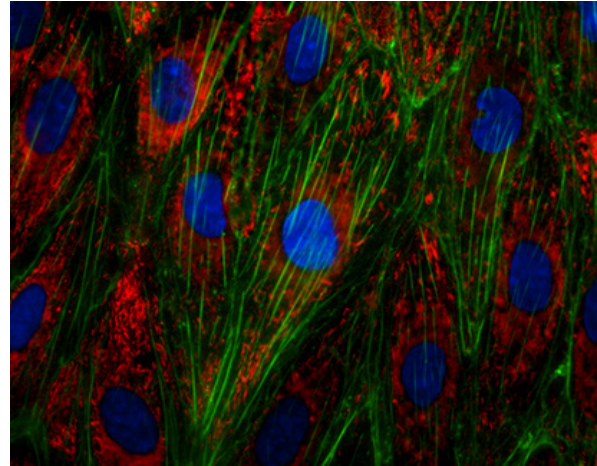
Immunofluorescence (IF) is a powerful approach for getting insight into cellular structures and processes using microscopy. Specific proteins can be assessed for their expression and location, making immunofluorescence indispensable for scientists to solve many cell biological questions.

An immunofluorescence experiment is based on the following principal steps:

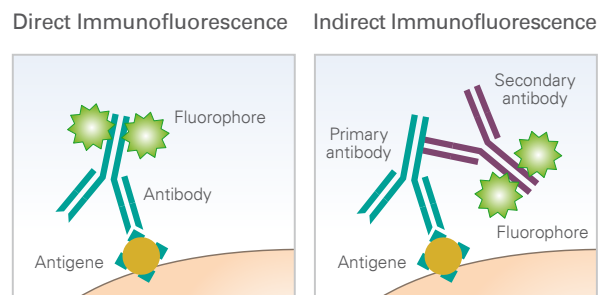
1. Specific antibodies bind to the protein of interest.
2. Fluorescent dyes are coupled to these immune complexes in order to visualize the protein of interest using microscopy.

It is distinguished between direct and indirect immunofluorescence. In direct immunofluorescence, the primary antibody is directly coupled to a fluorophore (also called fluorochrome), allowing for easy handling and quick visualization. In indirect immunofluorescence, a secondary fluorophore-coupled antibody, which specifically binds to the primary antibody, is used to visualize the structure of interest.

Although the second approach is more time-consuming than direct immunofluorescence, it has several big advantages, such as it is generally less expensive, because the secondary antibody can be used for different primary antibodies. In addition, several proteins can be specifically visualized in parallel in one single sample (multicolor immunofluorescence) by combining multiple primary antibodies with specific secondary antibodies—each of them labeled with a different fluorophore.



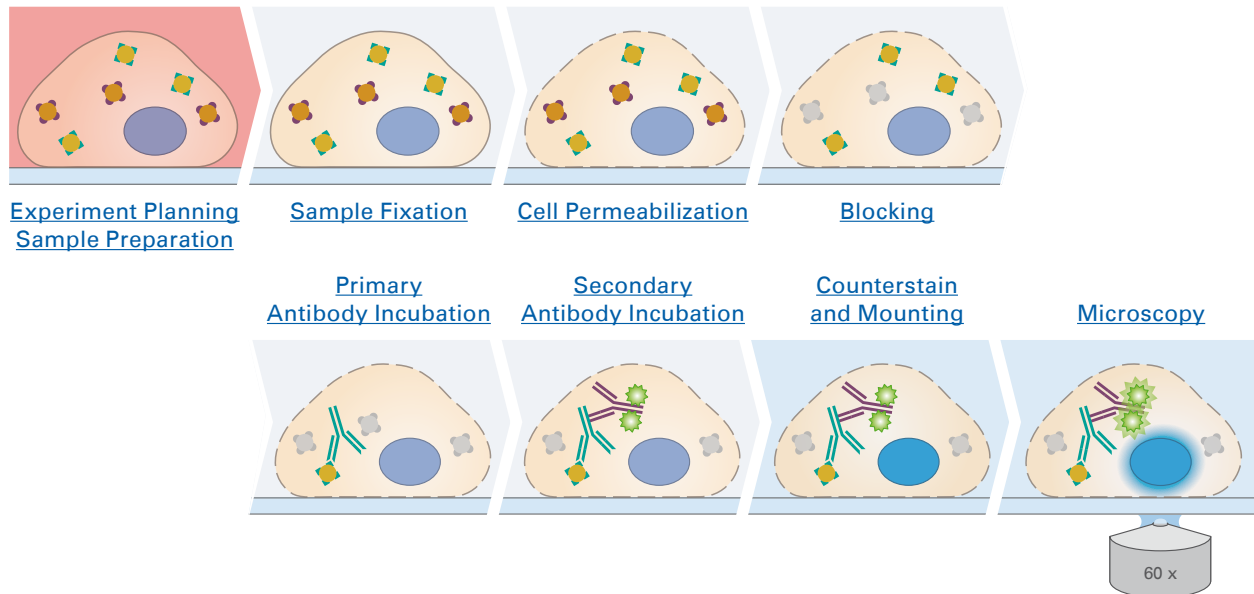
*Immunofluorescence staining of the von-Willebrand-Factor (vWF) in endothelial cells (HUVECs). Actin was stained using phalloidin (green), nuclei are stained with DAPI (blue).*



*The principle of direct immunofluorescence and indirect immunofluorescence.*

# Immunofluorescence Staining: A Typical Workflow

Every immunofluorescence staining protocol consists of four major steps (cultivation, fixation, staining, imaging), which can be subdivided as follows:



Immunofluorescence staining is a very sensitive method that might require [troubleshooting](#). Slight changes in the protocol can lead to different results that are no longer comparable. Therefore, it is very important to precisely maintain the exact same conditions in your specific protocol (e.g., cell density, antibody dilution, incubation temperature, and incubation time). The following is an overview of the different steps of an [indirect immunofluorescence](#) staining protocol.

## ibidi Solutions

The ibidi [μ-Slides and μ-Dishes](#) with a [coverslip bottom](#) for inverted microscopy are available in a variety of geometries that will fit any of your specific needs for immunocytochemistry. All immunofluorescence staining steps can be performed directly in the slides or dishes.



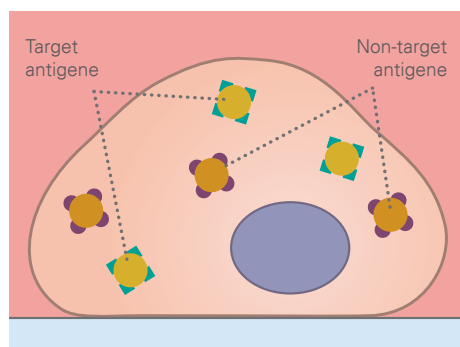
The geometry of the ibidi [Channel Slides](#) is ideal for the exact exchange of small medium amounts, which is necessary during immunocytochemistry stainings. In addition, the coverslip bottom of the channel μ-Slides eliminates the need for additional coverslips.

In the ibidi [Chamber Slides](#), a silicone gasket with separated chambers is mounted on a standard glass slide. The slides are ideal for the long-term storage of samples that are mounted with a glass coverslip.

Unlike most plastic materials used for cell culture, the ibidi labware is compatible with standard fixation methods. For a full overview, please check out our [chemical compatibility table](#). All immunofluorescence staining steps can be performed directly in the slides or dishes.

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## Experiment Planning and Sample Preparation



Before starting an immunostaining, a literature research should be done to determine the expression levels and intracellular localization of the protein of interest in the chosen model system. Some proteins have a very low expression, either generally, or in certain cell lines. In this case, the expression might have to be induced by external stimuli or overexpression techniques.

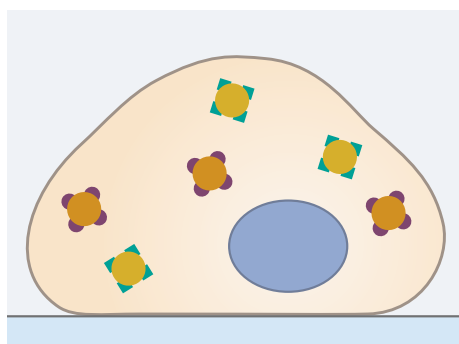
Further, the optimal cell density has to be determined. In general, a confluence of 70%–80% is recommended for IF.

In addition, the ideal cell culture vessel geometry and substrate/coating must be determined, and needs to be compatible with the chosen microscopy method. During the whole experiment, it is crucial that the cells never dry out, which should be considered when choosing the appropriate cell culture vessel geometry.

Finally, the number of samples to be stained and analyzed for statistical significance, including the appropriate controls, should be planned in advance.

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## Sample Fixation

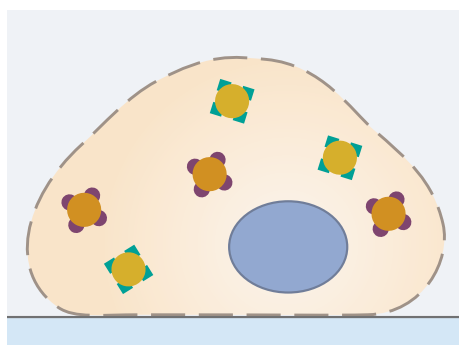


The first step of an immunofluorescence staining protocol is to fixate the sample. This is usually done by incubating the sample for 10 minutes at room temperature in a 4% formalin solution (in PBS, pH 7.4), which crosslinks the proteins. The sample can also be fixed in 100% chilled methanol or acetone.

Optimal fixation conditions should be determined individually for each experiment. Some proteins are destroyed by methanol fixation, and some antibodies do not detect proteins in formalin-fixed samples. After fixation, it is very important to wash the sample 3x for 5 minutes in a washing solution, (e.g., PBS) to remove the fixation solution completely.

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## Cell Permeabilization



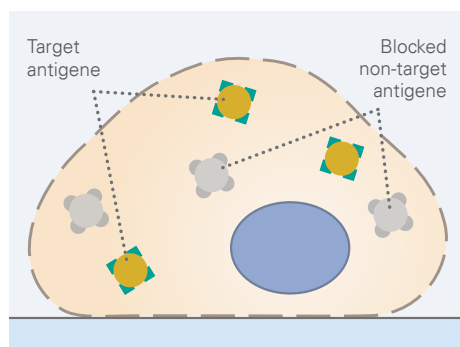
In order to stain intracellular proteins, the cell needs to be permeabilized. Without this step, it is not possible for the antibodies to enter the cell through the lipid membrane. The permeabilization requires incubation in a detergent, for example Triton X-100 or Tween-20 (for a less harsh permeabilization) in a PBS solution.

This step must be optimized depending on the protein of interest, the used antibody, and the experimental conditions. Especially when staining membranous proteins, the permeabilization step has to be done with caution, because Triton X-100 can destroy the cell membrane. In this case, using saponin might be an alternative to classic detergents. Don't forget that methanol-fixed samples are already permeabilized, since alcohols easily wash out the lipids of the cell membrane.

After this step, the sample has to be washed 3x for 5 minutes in a washing solution.

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## Blocking

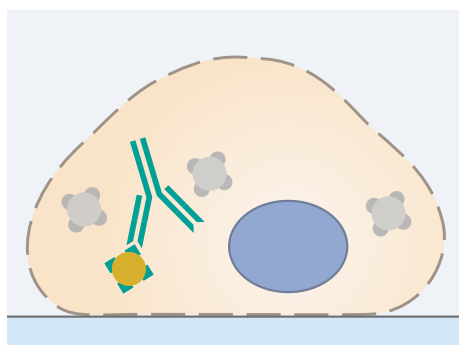


In order to minimize intra- or extracellular background signals, non-specific antigens should be blocked by incubating the sample in (1) the serum of the host, in which the secondary antibody was made, (2) bovine serum albumin (BSA), or (3) milk. The first option is the most recommended because of its highest specificity.

Typical blocking times are from 30 minutes up to one hour. Excessively long blocking steps should be avoided, as this can reduce the specific binding of the primary antibody, and therefore reduce the signal.

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## Primary Antibody Incubation



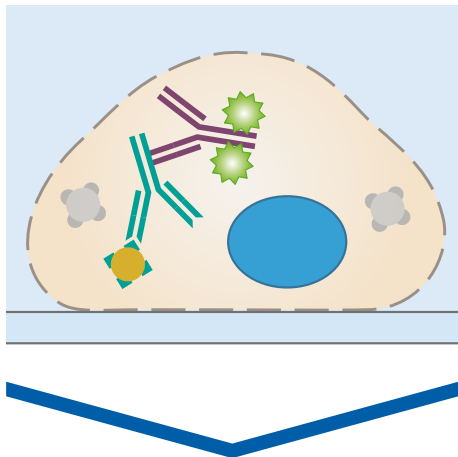
The selection of the primary antibody and its incubation conditions is the most critical step of an immunofluorescence staining protocol. Especially, if the protocol has not yet been established in the lab, a literature research is crucial. A suitable primary antibody must have a high specificity for the antigen of interest. Furthermore, whether the antibody is mono- or polyclonal influences its specificity. Many antibody manufacturers list references on their product pages, in which the antibody was successfully used in an immunofluorescence staining.

Another highly relevant property of the primary antibody is its originating host, as it determines the secondary antibody. This is especially important when doing multicolor stainings, because different specific primary antibodies for the parallel detection of several antigens in the same sample must be produced in different hosts, in order to avoid cross-reactivity.

The optimal incubation conditions must be determined carefully for each experiment. Antibody concentrations that are too high and incubation times that are too long can result in an unspecific background signal. Conversely, concentrations that are too low and incubation times that are too short can lead to a very weak or missing signal.

After incubation with the primary antibody, the samples should be washed 3x for 5 minutes in a washing solution in order to avoid background fluorescence.

## Secondary Antibody Incubation



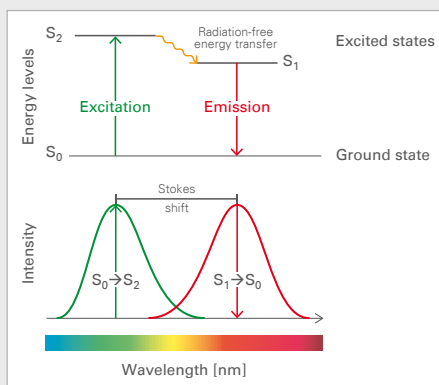
In standard immunofluorescence assays, the secondary antibody is conjugated to a fluorophore, which emits light when excited at a defined wavelength. The secondary antibody specifically binds to the first antibody. Therefore, it is essential that the secondary antibody is specific to the host in which the primary antibody was produced. For a successful experiment, the chosen microscopy technique and the equipment (filters, lasers, cameras, and detectors) also need to be compatible with the chosen fluorescence dye.

To be suitable for fluorescence microscopy, stable fluorophores must be used, because the sample is generally exposed to a high number of photons. Also, fluorophore brightness should be taken into account, because the antigen with the highest expression level should be detected with the fluorophore with the lowest brightness. When doing a multicolor staining, the spectral overlap of the conjugated fluorophores should be carefully considered as well. Respective tables can be found on the manufacturers' websites.

The incubation in the secondary antibody solution should be carried out according to manufacturer's protocol. Since fluorophores are sensitive to light, from now on all steps of the protocol need to be carried out in the dark.

In order to avoid background fluorescence, the samples should be washed 3x for 5 minutes in a washing solution after incubation with the secondary antibody.

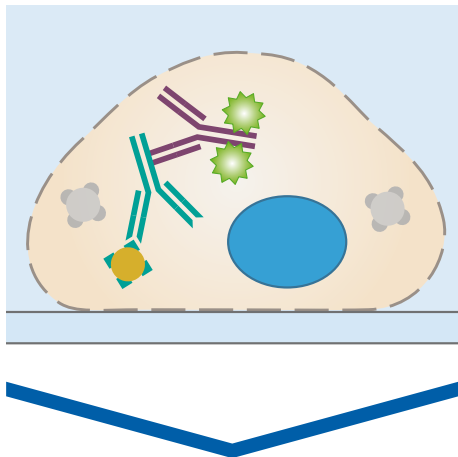
### Excuse: What is a fluorophore?



A fluorophore (also called fluorochrome) is a chemical molecule, which is used to label specific structures for microscopic analysis. For example, the green fluorescent protein (GFP), red fluorescent protein (RFP), and yellow fluorescent protein (YFP) are widely-used fluorophores in research.

Fluorophores are excited when they absorb light of a certain wavelength, leading to re-emission of light at a defined higher wavelength. A successful excitation by light transfers the fluorophore from the ground state ( $S_0$ ) to an excited state ( $S_2$ ). This high-energy state is not stable and quickly undergoes changes. The fluorophore vibrates, leading to light release and a return to the ground state after a few picoseconds. The vibrations in the excited states cause the so-called Stokes shift, which means that the wavelength of the emitted light is always higher than that of the excitation light.

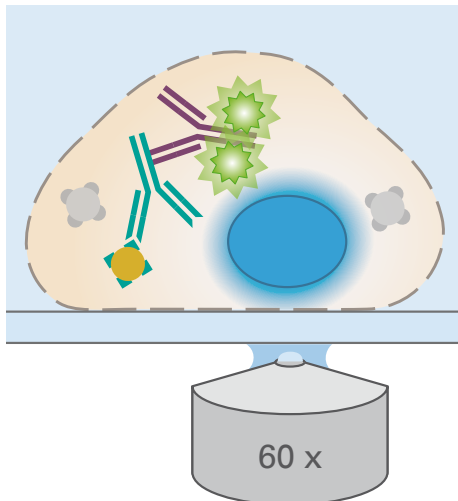
## Counterstain and Mounting



The final step before microscopy is the counterstaining of the nuclei and the mounting. In order to avoid drying out of the samples, and to guarantee a stable refractive index of the cellular environment (a prerequisite for successful microscopy), the sample needs to be mounted.

For this, the sample should be covered with a mounting medium that has a low autofluorescence. DAPI, the standard for nuclear counterstaining, is either included in the mounting medium, or can be added separately.

## Microscopy



To get optimal results, the microscopic analysis of the immunofluorescence staining should be done directly after the mounting.

Many microscopy techniques exist, each optimized for different experimental approaches. For standard immunofluorescence stainings, [epifluorescence](#) and [confocal microscopy](#) are widely-used methods. Of course, all parameters such as magnification and exposure should be carefully and individually determined.

Please find a detailed overview of the different microscopy techniques and their applications [on our website](#), or download the "[Microscopy With the ibidi Chambers](#)" Application Guide as a PDF.

### ibidi Solutions

The [ibidi Mounting Medium](#) and the [ibidi Mounting Medium With DAPI](#) have a very low autofluorescence, prevent photobleaching and allow the sample to be stored for several weeks on the  $\mu$ -Slide without the need for additional coverslips.

If your experiment requires long-term storage of immunostained samples, we recommend using the ibidi [Chamber Slides, removable](#). Since signals could fade if fluorophores are exposed to light for a longer period, the samples always must be stored in the dark.



## Troubleshooting

Immunocytochemistry is a very sensitive method that requires a lot of experience and optimization. Slight changes in the protocol can markedly alter the results. If you get a low signal, no signal, or a high background, please consult the following troubleshooting guide.

### High Background

Reason	Solution
Inappropriate or too long fixation, leading to artefacts	Reduce fixation time or change the fixative
Insufficient blocking	Prolong the incubation time, consider using a different blocking solution (we recommend using serum from the secondary antibody host)
No specificity of the primary antibody	Use a primary antibody that is proven to work for immunofluorescence in the chosen model system; if available, use a knockdown/knockout sample as a negative control
Too high primary/secondary antibody concentration, too long incubation time, too high incubation temperature	Optimize the antibody concentration and incubation time/temperature, consult manufacturer's protocol
Cross-reactivity of the secondary antibody	Use isotype controls for the secondary antibody to check for cross-reactivity
Not enough washing	Verify that all washing steps are carried out properly; if necessary, prolong the washing steps
Bleaching during imaging	Use a secondary antibody conjugated to a fluorophore suitable for your chosen microscopy technique
Low signal intensity, resulting in noise	Optimize the signal-to-noise ratio, e.g., by using a brighter fluorophore for detection; if applicable, increase the expression of the antigen of interest (e.g., by overexpression or by addition of inducing agents)
High autofluorescence	Check the sample autofluorescence by using unstained controls; use fresh fixation solutions (expired formalin solutions might have a high autofluorescence); use materials with low autofluorescence (e.g., <a href="#">ibidi µ-Slides</a> or <a href="#">Chamber Slides</a> ); use mounting medium with low autofluorescence (e.g., <a href="#">ibidi Mounting Medium</a> / <a href="#">ibidi Mounting Medium With DAPI</a> )

### Low Signal or Lack of Signal

Reason	Solution
Drying out of the sample	Always keep the sample moist
Overfixation of the sample, leading to epitope damage	Reduce fixation time or change the fixative
Inadequate permeabilization method	Optimize or skip the permeabilization step
No binding of primary antibody to the antigen of interest	Use a primary antibody that is proven to work for immunofluorescence in the chosen model system; check the antibody functionality by using a positive control (e.g., by overexpression or by addition of inducing agents)
Too high primary/secondary antibody dilution, too short incubation time, too low incubation temperature	Increase the antibody concentration or the incubation time/temperature; consult manufacturer's protocol
Very low or no antigen expression	Use a positive control (e.g., an overexpression model); reconsider your experimental system
Inappropriate microscopy detection method	Use a more sensitive method for image acquisition; check your used filter/laser setup; use a brighter fluorophore for detection

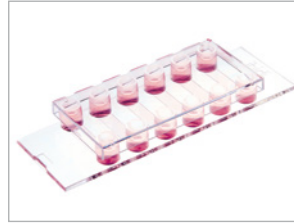


# Immunofluorescence With the ibidi Chambers

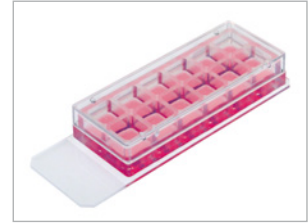
ibidi provides several solutions that fit your needs for immunofluorescence assays:



[Chambered Coverslips](#)



[Channel Slides](#)



[Chamber Slides, removable](#)

Bottom material	<a href="#">Glass Coverslip</a> or <a href="#">Polymer Coverslip</a>	<a href="#">Glass Coverslip</a> or <a href="#">Polymer Coverslip</a>	Standard glass slide
Additional coverslips required?	No	No	Yes (for mounting)
Microscope type	Inverted	Inverted	Inverted & upright
Mounting medium	Non-hardening	Non-hardening	Hardening
Sample storage	Short-term	Short-term	Long-term

## Benefits of ibidi $\mu$ -Chambers

### 1. High-resolution imaging

The ibidi slides are ideal for [widefield fluorescence](#), [confocal imaging](#), [FRAP](#), [FRET](#), [FLIM](#), and undisturbed [phase contrast imaging](#).

### 2. Fast and simple handling

The all-in-one chambers simplify your immunofluorescence protocol.

### 3. Cost-effective experiments

Only a small number of cells and low reagent volumes are needed.

With their thin coverslip bottom, the ibidi  [\$\mu\$ -Dishes](#) and  [\$\mu\$ -Plates](#) are also ideally suitable for immunofluorescence stainings and high-resolution microscopy.



Find detailed immunofluorescence staining protocols in the ibidi Application Notes:

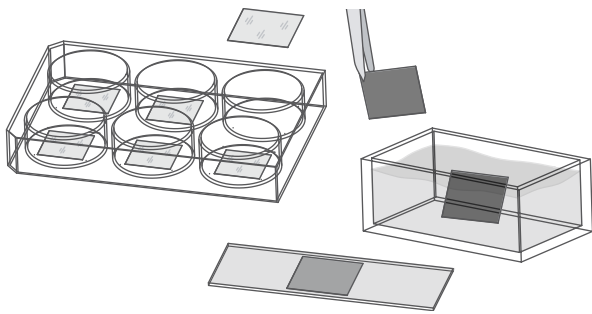
- [AN 02: Fluorescence Staining using a  \$\mu\$ -Slide I \(PDF\)](#)
- [AN 09: Fluorescence Staining using a  \$\mu\$ -Slide VI <sup>0.4</sup> \(PDF\)](#)
- [AN 15: Fluorescence Staining using a  \$\mu\$ -Slide  \$\gamma\$ -shaped \(PDF\)](#)
- [AN 16: Fluorescence Staining using a  \$\mu\$ -Slide 8 Well \(PDF\)](#)
- [AN 49: Fluorescence Staining using a 12 Well Chamber, removable \(PDF\)](#)
- [AN 50: Fluorescence Staining using a 3 Well Chamber, removable \(PDF\)](#)
- [AN 45: Mounting Medium Types \(PDF\)](#)

## Comparison of Immunocytochemistry Protocols: Traditional Staining vs. Staining With ibidi Solutions

When using any of the ibidi solutions, the immunofluorescence staining protocol is much shorter than the traditional protocol. There is no need to grow the cells on loose coverslips—the cells can be grown and stained directly in the ibidi slides.

### Protocol With Cells on Coverslips

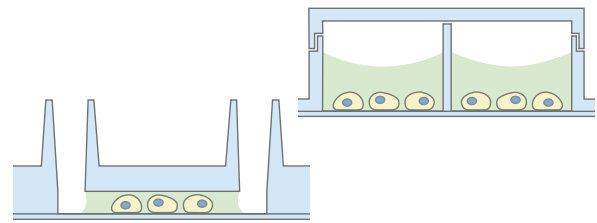
Traditional method with nail polish mounting



- Sterilize coverslips and slides
- Coat coverslips
- Place sterile coverslips into 6-well plate
- Seed cells in large volume
- Peel off the coverslip
- Wash
- Fix – wash – permeabilize – wash – block
- Incubate in primary antibody – wash – incubate in secondary antibody
- Wash
- Mount cells with mounting medium
- Mount coverslip with nail polish

### Protocol With ibidi $\mu$ -Slides

Time-saving method using all-in-one chambers



- Sterilize coverslips and slides
- Coat coverslips
- Place sterile coverslips into 6-well plate
- Seed cells in large volume
- Peel off the coverslip
- Wash
- Fix – wash – permeabilize – wash – block
- Incubate in primary antibody – wash – incubate in secondary antibody
- Wash
- Mount cells with mounting medium
- Mount coverslip with nail polish

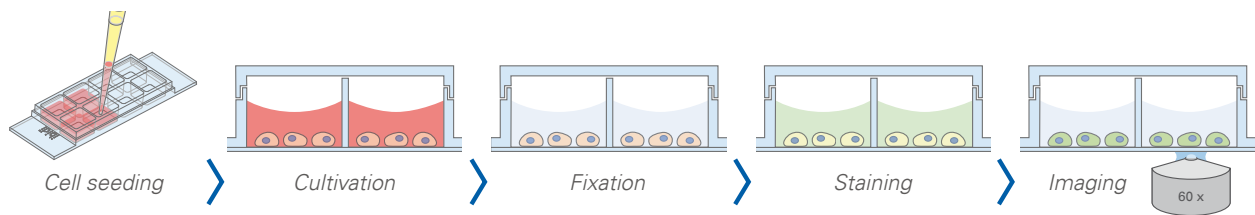
### ibidi Solutions

The [ibidi Mounting Medium](#) and the [ibidi Mounting Medium With DAPI](#) have a very low autofluorescence, prevent photobleaching and allow the sample to be stored for several weeks on the  $\mu$ -Slide without the need for additional coverslips.

If your experiment requires the long-term storage of immunostained samples, we recommend using the [ibidi Chamber Slides, removable](#).



## Chambered Coverslips



When using chambered coverslips, such as the ibidi [μ-Slides](#), the cell culture and the whole immunofluorescence staining protocol can be performed in the same well, without the necessity of any additional coverslips or tweezers. After mounting, the results can be observed through the coverslip bottom using high-resolution microscopy.

### Advantages

- No coverslip handling
- Parallel assays without cross-contamination

### Limitations

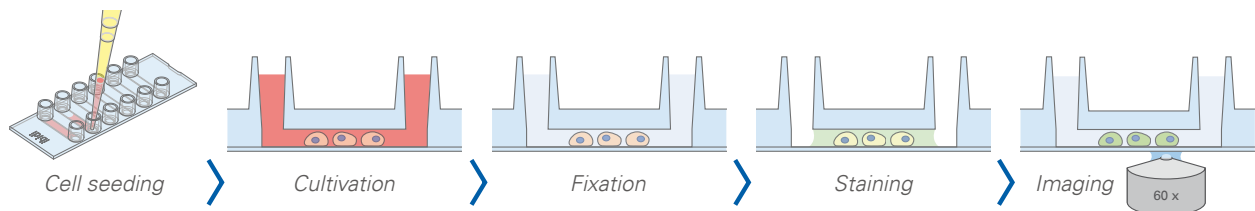
- Storage time is restricted to several weeks, because of the gas exchange through the plastic material from which the slides are made

Find more information and technical details about the coverslip bottom of the ibidi chambers [on our website](#).

The ibidi [μ-Slides](#) are available in a variety of well numbers, volumes, and geometries that fit your specific experiment.



## Channel Slides



The ibidi channel slides are particularly suitable for immunofluorescence stainings: The geometry of the ibidi [Channel Slides](#) is ideal for the exact exchange of small medium amounts, which is necessary during immunocytochemistry stainings. In addition, the coverslip bottom of the channel μ-Slides eliminates the need for additional coverslips.

### Advantages

- Meniscus-free phase contrast microscopy
- Low volumes of reagents needed
- Homogeneous cell and antibody distribution

### Limitations

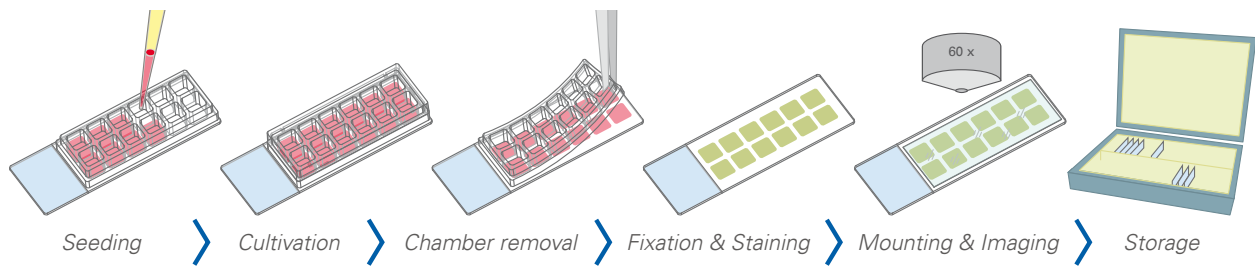
- Storage time is restricted to several weeks, because of the gas exchange through the plastic material from which the slides are made

Find more information and technical details about the coverslip bottom of the ibidi chambers [on our website](#).

The [μ-Slide VI<sup>0.4</sup>](#) is ideal for general low-volume immunofluorescence assays. The [μ-Slide I Luer](#) enables immunofluorescence staining in low-density cultures in the context of flow experiments.

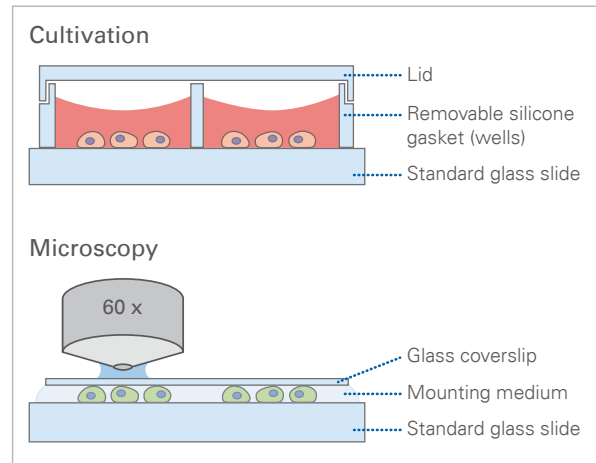
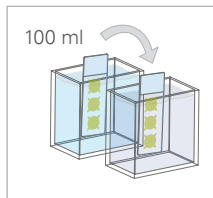


## Chamber Slides



In the ibidi [Chamber Slides](#), a silicone gasket with separated chambers is mounted on a standard glass slide. The ibidi Chamber Slides for immunofluorescence are ideal for the long-term storage of samples that are mounted with a glass coverslip.

For high-throughput approaches, the silicone gasket can be removed before starting the staining. This allows for the processing of several slides at once in a staining jar. As an alternative to low-throughput experiments, the silicone gasket can be left in position, allowing for separate staining in each well.



### Advantages

- Ideal for long-term storage
- High-throughput screening possible

### Limitations

- No high-resolution microscopy during cell cultivation

The ibidi Chamber Slides, removable are available with [3 wells](#), [8 wells](#), and [12 wells](#).



## Immunofluorescence Applied: Experimental Examples

- [Visualization of Endothelial Cell Junctions](#)
- [Immunostaining of Rat Dorsal Root Ganglionic Cells and Schwann Cells](#)
- [Rat Hippocampal Neuron and Astrocyte Staining](#)
- [Adherens Junctions and Actin Cytoskeleton of HUVECs Under Flow](#)
- [Mitochondria Staining of MDCK cells](#)
- [Focal Adhesions of Differentiated Mouse Fibroblasts on an Elastic Surface](#)

### Visualization of Endothelial Cell Junctions

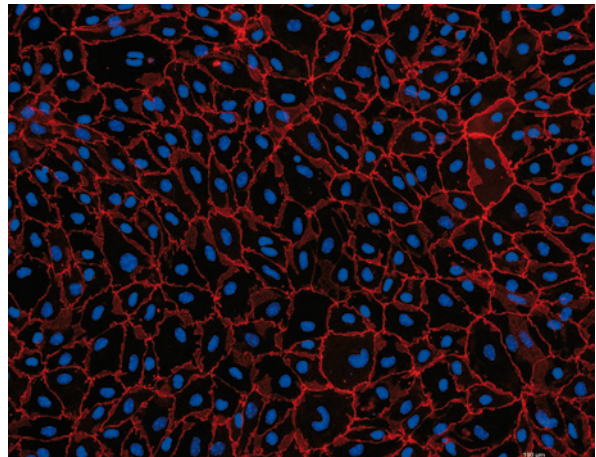
Immunofluorescence staining of primary mouse brain microvascular endothelial cells (pMBMECs), cultured in the ibidi [12 Well Chamber, removable](#). Red color delineates the endothelial cell junctions labeled for zonula occludens (ZO)-1, highlighting their matured state. Blue color shows the nuclei stained with DAPI. The image was acquired with a 20x objective on a Nikon Eclipse microscope.

*Data by Sidar Aydin, Britta Engelhardt, Ruth Lyck, Theodor Kocher Institute, Bern, Switzerland.*

ibidi product used:



[12 Well Chamber, removable](#)



### Immunostaining of Rat Dorsal Root Ganglionic Cells and Schwann Cells

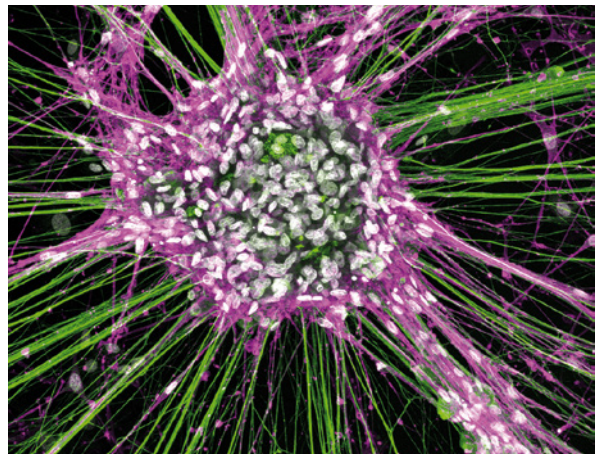
Rat dorsal root ganglionic cells and Schwann cells cultured in an ibidi [μ-Slide 8 Well](#) and stained for neurofilament (green), NGFR (magenta), and DAPI (white). The image was obtained with a LEICA SP8X laser scanning microscope.

*Data by Tamara Weiss, Division of Plastic and Reconstructive Surgery, Medical University of Vienna, Austria.*

ibidi product used:



[μ-Slide 8 Well](#)



## Rat Hippocampal Neuron and Astrocyte Staining

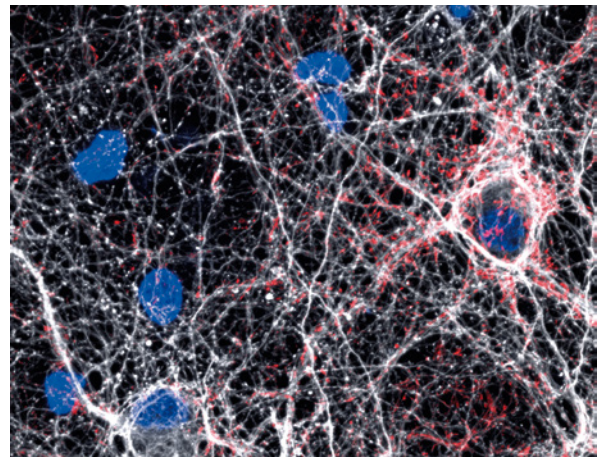
Confocal microscopy of rat hippocampal neurons plated over astrocytes, cultured on ibidi [μ-Plate 24 Well](#). Red is Synapsin 1, a pre-synaptic marker, white is neuron-specific Tubulin Beta-III, and blue is DAPI, a nuclear counterstain.

Data by Daniel Hoepfner, Lieber Institute for Brain Development, Baltimore, MD, USA.

ibidi product used:



[μ-Plate 24 Well](#)



## Adherens Junctions and Actin Cytoskeleton of HUVECs Under Flow

Human umbilical vein endothelial cells (HUVECs) were cultured under [flow conditions](#) in the ibidi [μ-Slide I<sup>0.4</sup> Luer](#).

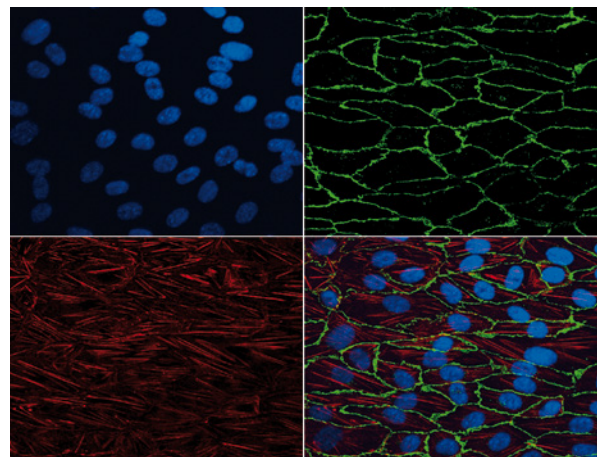
Red: actin cytoskeleton (Cy5-conjugated antibody).  
Green: adherens junctions, marked by VE-cadherin (Alexa 488-conjugated antibody).  
Blue: nuclear counterstaining (DAPI).

Data by S. Zahler, Munich, Germany.

ibidi product used:



[μ-Slide I<sup>0.4</sup> Luer](#)



## Mitochondria Staining of MDCK cells

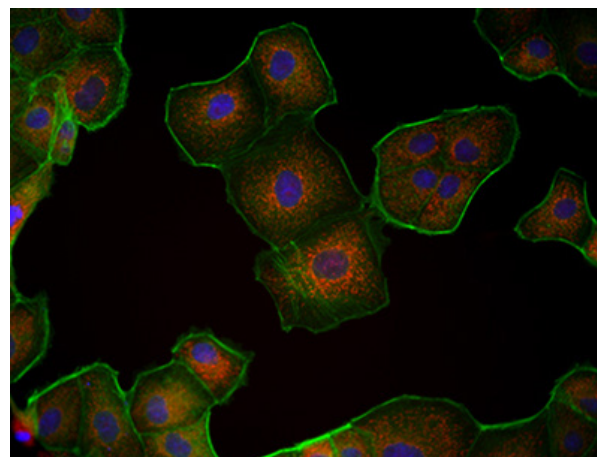
Madin-Darby canine kidney (MDCK) cells were cultured in the ibidi [μ-Slide VI<sup>0.4</sup>](#).

Green: F-actin cytoskeleton (Alexa488-Phalloidin).  
Red: mitochondria (MitoTracker).  
Blue: nuclear counterstaining (DAPI). Widefield fluorescence using 100x objective lens with oil immersion.

ibidi product used:



[μ-Slide VI<sup>0.4</sup>](#)

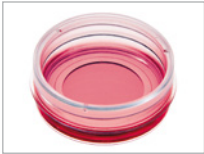


## Focal Adhesions of Differentiated Mouse Fibroblasts on an Elastic Surface

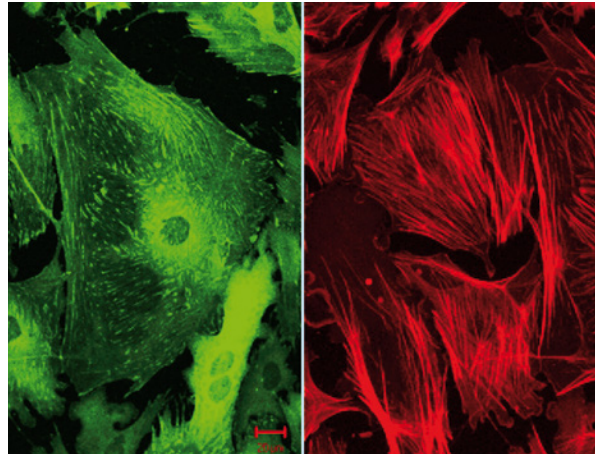
Differentiated mouse fibroblasts were cultured on an elastic surface using the [μ-Dish<sup>35 mm, high ESS</sup>](#) (28 kPa). Green: focal adhesions, marked by zyxin (Alexa 488-conjugated antibody). Red: alpha-smooth muscle actin (Cy5-conjugated antibody).

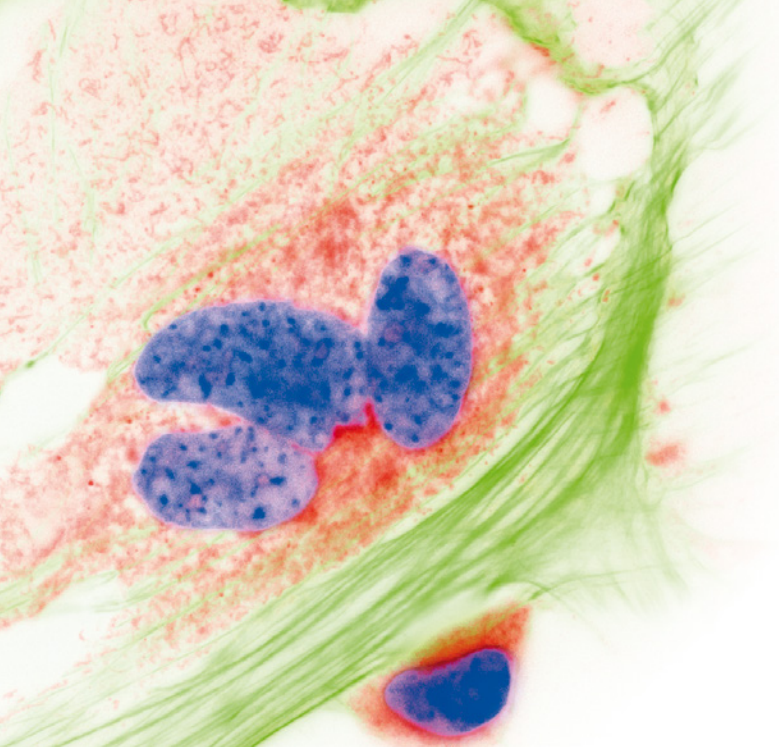
*Data by R. Merkel, Jülich, Germany.*

ibidi product used:



[μ-Dish<sup>35 mm, high ESS</sup>](#)





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