

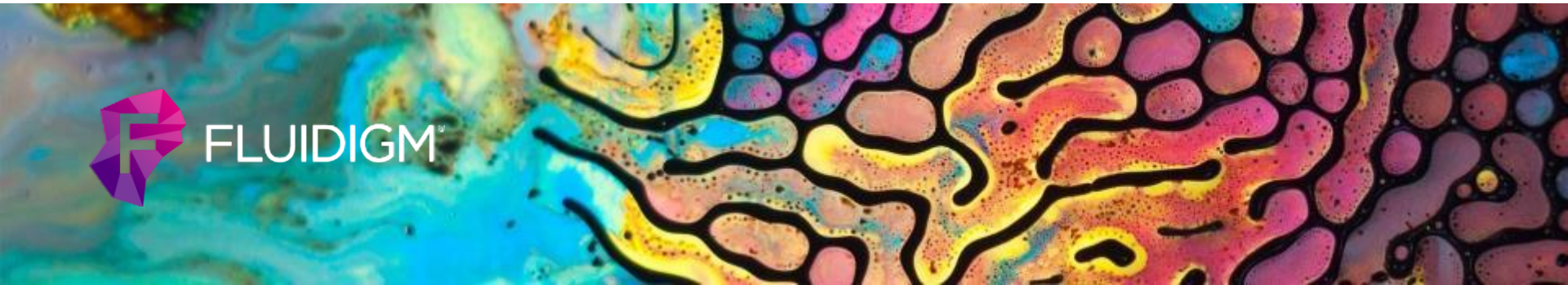
# IMC Experiment Workflow

Xinlei Chen

2020-4-13



FLUIDIGM™



# Hyperion Imaging System



## Hyperion 实验开展技术要点

2020-02-26 Version 2.1

### 前言

Hyperion 组织成像质谱流式系统由成像质谱流式技术 (Imaging Mass Cytometry) 发展而来, 可以实现对组织石蜡切片、冰冻切片等样本进行几十个参数的同时检测, 在组织微环境相关研究中发挥着重要作用。在这里, 我们通过查阅相关的文献, 以及组织微环境技术支持团队及部分客户分享的经验, 对 Hyperion 实验的流程及技术细节进行梳理和总结, 希望可以帮助大家快速掌握相关的技术要领, 顺利开展相关实验。

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# IMC Experiment Workflow

一、切片样本的准备和储存

二、实验设计

三、Panel验证（即预实验）

四、正式实验

五、数据分析

# 切片的选择

	冰冻切片	石蜡切片
制片过程	制样简单，将新鲜组织用OTC包埋速冻、切片即可，对实验人员技术有一定要求	步骤较多，固定过夜，包埋，切片等步骤
染色过程	步骤简单，固定，打孔，封闭后即可染抗体	需要脱蜡、复水、抗原修复等操作
抗体选择	有38种IMC预标记抗体可供选择，但第三方抗体选择相对容易，需要选择IHC-F的抗体	目前有88种IMC预标记抗体，第三方抗体选择需要IHC-P的切片，并要求抗原修复条件的一致性
形态保存	组织结构易变形	组织结构保存良好
样本来源	临床上存量较少，往往需要从新鲜组织制备	大量包埋好的组织蜡块，可以支持长期的回溯性研究
样本保存	组织块和切片均需要冻存在-80度	组织块：常温放置；切片：需要石蜡油密封，四度保存

冰冻切片和石蜡切片的比较



劣势



优势

# 切片的制备

组织样本处理、切片等步骤，与免疫组化制备方法相同

- **FFPE切片**需要经过中性甲醛固定、石蜡包埋、切片等步骤
- **冰冻切片**则需要OCT包埋剂中将组织速冻，冰冻切片等步骤
- Hyperion实验切片厚度要求 $\leq 7\mu\text{m}$ ，文献中最常用的厚度：**5 $\mu\text{m}$**

# 切片的保存

良好的切片质量，对于获得高质量的实验数据至关重要

## 冰冻切片

包埋冷冻好的组织块和切片都可以在-80保存一年。切片样本需要装在密封的盒子中冻存，取出切片时注意先平衡温度至室温后再打开盒子，以免生成冷凝水。

# 切片的保存

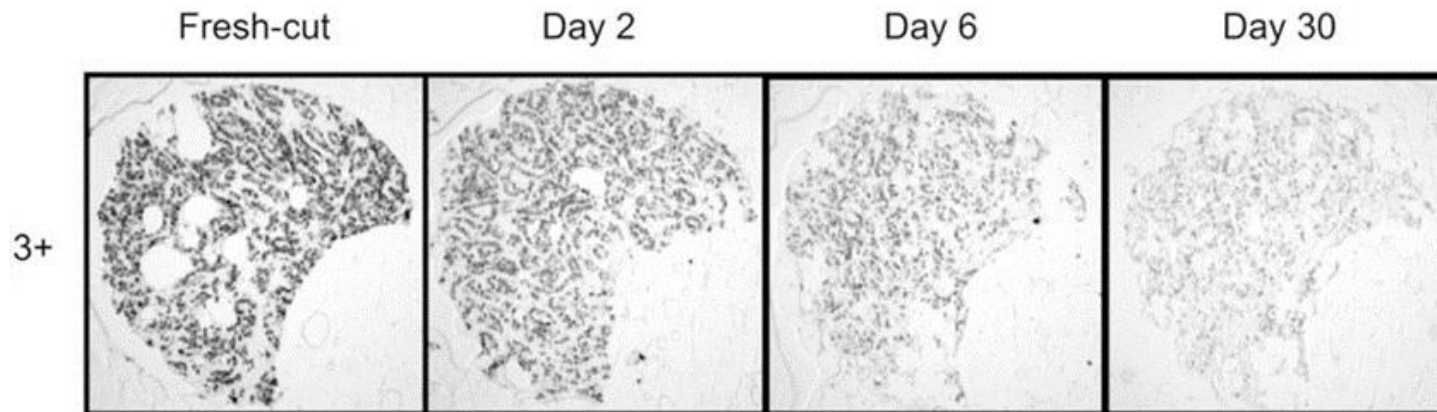
## 石蜡切片

- **影响因素**：空气（引起氧化）、水（引发水解）以及阳光（温度以及辐射的影响）等
- **石蜡块**中的组织由于有包埋石蜡的阻隔，处于相对封闭的状态，一般情况下可以在常温保存数年。
- **石蜡切片**，组织已经被暴露出来，保存的条件、抗体种类都有可能对结果产生很大影响。因此，我们强烈建议对研究项目进行良好的设计和规划，尽可能多的使用Fresh Cut的切片。

# 切片的保存

## 石蜡切片

1) 在毫无保护的情况下常温放置，会迅速导致部分抗原信号的降低、丢失。





# 切片的保存

## 石蜡切片

2) 在文献中验证的保存方法有以下策略:

A 充氮隔绝空气(Nitrogen Storage)

B 石蜡封闭(Paraffin coating);

C 降低温度 (4度保存) ;

*由于不同抗原性质存在非常大的差异，使用单一策略可能效果不佳，文章里更多的是验证这些策略的组合以提高抗原信号的保存程度*

# IMC Experiment Workflow

一、切片样本的准备和储存

**二、实验设计**

三、Panel验证（即预实验）

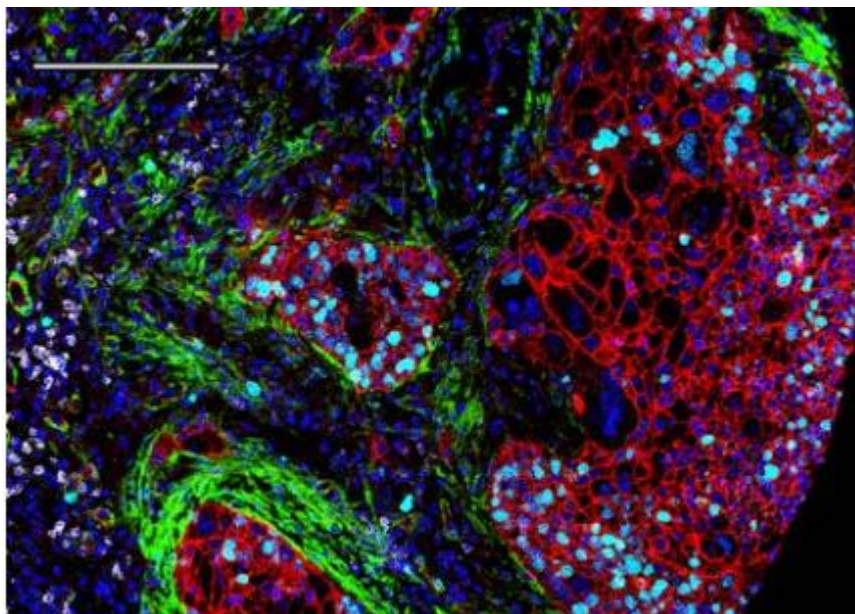
四、正式实验

五、数据分析

# 1、检测靶标蛋白的筛选

## 1.1 组织结构相关蛋白

	Antibody	Metal Tag
Structural	Alpha-smooth muscle actin (SMA)	141Pr
	Vimentin	143Nd
	Collagen type I	169Tm
	Beta-catenin	165Ho
	E-cadherin	158Gd
Cancer	HMWK	144Nd
	Ki-67	168Er
	p53	150Nd
Immuno-oncology	CD45	152Sm
	CD3	170Er
	CD8a	162Dy
	CD20	161Dy
	CD68	159Tb
	PD1	164Dy
	CTLA4	161Dy
	CD31	145Nd
	Myeloperoxidase	146Nd
	p40	167Er
CD44	171Yb	
Nuclear		191Ir
		193Ir

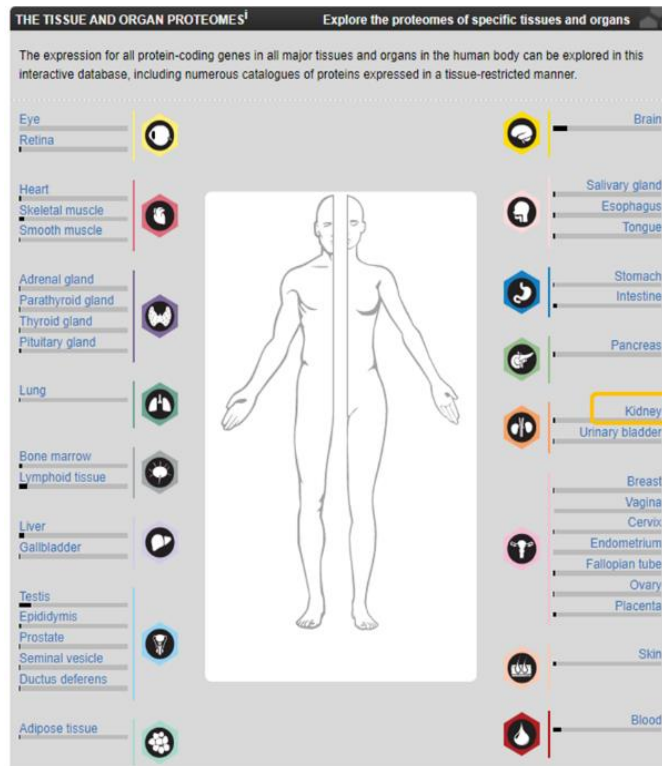


# 1、检测靶标蛋白的筛选

## 1.1 组织结构相关蛋白

**THE TISSUE AND ORGAN PROTEOMES<sup>1</sup>** Explore the proteomes of specific tissues and organs

The expression for all protein-coding genes in all major tissues and organs in the human body can be explored in this interactive database, including numerous catalogues of proteins expressed in a tissue-restricted manner.



Eye  
Retina

Heart  
Skeletal muscle  
Smooth muscle

Adrenal gland  
Parathyroid gland  
Thyroid gland  
Pituitary gland

Lung

Bone marrow  
Lymphoid tissue

Liver  
Gallbladder

Testis  
Epididymis  
Prostate  
Seminal vesicle  
Ductus deferens

Adipose tissue

Brain

Salivary gland  
Esophagus  
Tongue

Stomach  
Intestine

Pancreas

**Kidney**

Urinary bladder

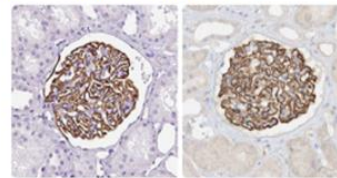
Breast  
Vagina  
Cervix  
Endometrium  
Fallopian tube  
Ovary  
Placenta

Skin

Blood

### Proteins specifically expressed in glomerulus

The process of urine formation begins in the glomerulus, where an ultrafiltrate of plasma is formed, and the filtered fluid enters the renal tubules. The filter consists of three layers; the fenestrated endothelium, the basement membrane, and the podocyte slit diaphragm. The analysis of the glomerulus elevated proteins is well in line with the function of the glomerulus as a filtration apparatus assembling a slit diaphragm. The list of kidney elevated proteins includes several well-known glomeruli associated genes, such as podocin (NPHS2) and nephrin (NPHS1), well established as proteins creating the filtration diaphragm making up a filter for large molecules. In addition, KIRREL1 is present in the glomerulus as described before. This latter protein is not identified as elevated in kidney, since the placenta shows higher mRNA levels than kidney for this gene. The placenta also acts as a filtration machinery for large and small molecules is therefore interesting. Another kidney elevated gene expressed in the glomerulus is FGF1.



NPHS2

NPHS1

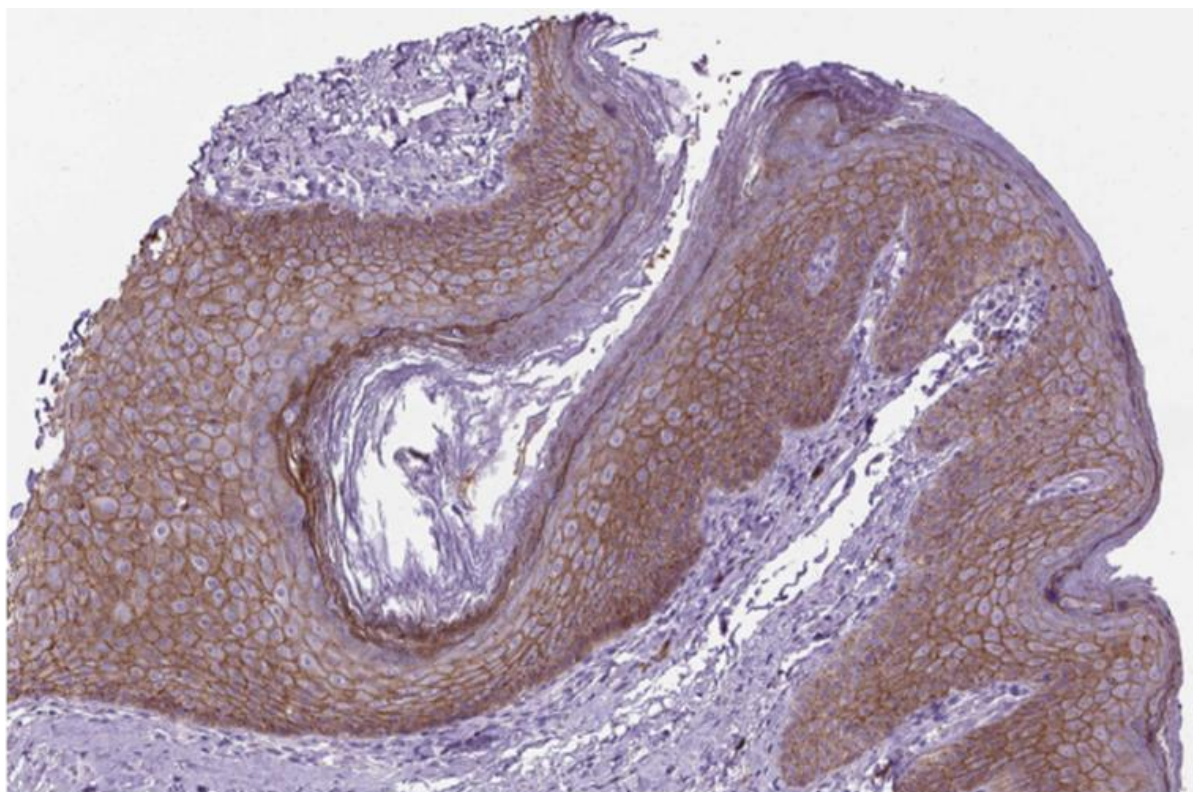
### Proteins specifically expressed in proximal tubule

Approximately 60% of the filtered  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{H}_2\text{O}$  and more than 90% of the filtered  $\text{HCO}_3^-$  are absorbed along the proximal tubule. This is also the segment that normally reabsorbs virtually all the filtered glucose and amino acids. An additional function is the secretion of numerous organic anions and cations. Most of the proteins elevated in the kidney are localized to the proximal tubule, which is in line with the function of proximal

网址: <https://www.proteinatlas.org/humanproteome/tissue>

# 1、检测靶标蛋白的筛选

## 1.2定位于细胞边缘的蛋白



如图：E-Cadherin在真皮细胞中的表达，显示出明显的膜定位

图片来源：<https://www.proteinatlas.org/>

对于后期图像处理  
时进行单细胞化非  
常有帮助

# 1、检测靶标蛋白的筛选

## 1.3与课题相关的目标蛋白

1) 做好信息收集，认真查阅每个蛋白在目标组织中的表达情况，包括表达强度、阳性率等情况。剔除组织不表达的目标蛋白，以防止后续的资源浪费。

对于人的样本，Protein ATLAS网站可以提供详细的参考资料。

2) 对于组织中阳性细胞非常稀少的marker，应谨慎处理，结合目标蛋白在课题中的重要性，决定是否保留。

3) 对于表达量很低的蛋白，也要谨慎处理。IMC实验需要很多抗体共用一个抗原修复条件，每个抗体不一定是在它的最适合条件下染色的，所以Panel中如果有过多的低表达目标蛋白会给后续的实验条件的优化带来一定困难。

## 2、抗体的选择

### 2.1 预标记抗体

尽量多的使用预标记抗体，可以简化预实验阶段的验证工作，提高实验的成功率。



Fludigm 预标记IMC抗体分类

*\*IMC抗体货号都以字母"D"结尾，规格为25ug，碱性抗原修复，全部是人的*

# 2、抗体的选择

## 2.1预标记抗体

小部分用于质谱流式悬液检测（Helios）抗体可以和冰冻切片反应。

如果手上已经有相关的抗体，可以自行进行validation，挑选出可以使用的部分；

如果手上没有，建议直接购买合适的第三方抗体进行标记。



# 2、抗体的选择

## 2.2第三方抗体

为了使金属Label反应正常进行，第三方抗体的要求具体如下：

- 抗体可以用于IHC-P或IHC-F（具体选择哪种，要根据样本种类决定）
- 抗体本身是裸抗，即不带有荧光或其他性质的标签；
- 抗体中不含有BSA、血清这些大分子添加物；
- 同一Panel抗原修复条件保持一致（仅对FFPE样本）

# 2、抗体的选择

## 2.2第三方抗体

### FFPE样本抗原修复条件的选择

*酸性修复 (citrate buffer pH6.0)*

*碱性修复(Tris/EDTA buffer pH9.0)*

- 在选择抗体的过程中需要向裸抗供应商询问其适合的抗原修复条件，务必保持一致，以便保证所有抗体均可以做出想要的信号。
- 优先选择那些在IMC文章中已经报道使用过的克隆号，这样可以提高后面实验的成功率。

### 3、金属标签匹配（配色）

- 1) 预标记抗体的标签种类，尽量多的使用预标记抗体，可以简化预实验阶段的验证工作，提高实验的成功率。
- 2) 通道之间的相互影响，这里主要指部分同位素的impurity导致的对邻近一些通道影响。这个影响比例虽然很小，但是应该避免把信号强度悬殊的marker安排在相关通道上。
- 3) 目标蛋白的表达强度，对于Hyperion来说在159~169之间是灵敏度最高的区域，因此可以考虑把较弱的抗体放在这个区域内。（关于目标蛋白的表达强度信息可以从ATLAS网站上获得，在查阅相关数据时注意选择匹配的组织类型。）
- 4) 使用镧系金属，总数35个

# 3、金属标签匹配 (配色)

## Panel Design Helper

IMC_test1		Human	IMC-FFPE										Load		Updates		Type
Markers	Tag	Custom	Tag1	Tag2	Tag3	Tag4	Tag5	Tag6	Tag7	Tag8	H_ATLAS	Intensit	Forker	Protocol	Type		
Alpha-SMA		<input type="checkbox"/>	141								<a href="#">ACTA2</a>			IMC-P			
Vimentin		<input type="checkbox"/>	143	143							<a href="#">VIM</a>			IMC-P			
Collagen type I		<input type="checkbox"/>	169								<a href="#">COL1A1</a>			IMC-P			
beta catenin		<input type="checkbox"/>	165								<a href="#">CTNNB1</a>			IMC-P			
E-Cadherin		<input type="checkbox"/>									<a href="#">CDH1</a>			IMC-P			
HMWK		<input checked="" type="checkbox"/>												IMC-P			
Ki-67		<input type="checkbox"/>	168								<a href="#">MKI67</a>			IMC-P	L		
p53		<input type="checkbox"/>	143								<a href="#">TP53</a>			IMC-P			
CD45		<input type="checkbox"/>	153								<a href="#">PTPRC</a>			IMC-P			
CD3		<input type="checkbox"/>	170								<a href="#">CD3G</a>			IMC-P			
CD8a		<input type="checkbox"/>	162	162							<a href="#">CD8A</a>			IMC-P			
CD20		<input type="checkbox"/>	161								<a href="#">MS4A1</a>			IMC-P			
CD68		<input type="checkbox"/>									<a href="#">CD68</a>			IMC-P			
PD1		<input type="checkbox"/>	148								<a href="#">PDCD1</a>			IMC-P			
CTLA4		<input type="checkbox"/>									<a href="#">CTLA4</a>			IMC-P			
CD31		<input type="checkbox"/>	151								<a href="#">PECAM1</a>			IMC-P			
CD44		<input type="checkbox"/>									<a href="#">CD44</a>			IMC-P			
CD56		<input type="checkbox"/>									<a href="#">NCAM1</a>			IMC-P			
CD19		<input type="checkbox"/>	142								<a href="#">CD19</a>			IMC-P			

链接: <https://pan.baidu.com/s/1ATFOLpsbW-UCcKpWGHmESQ> 密码: f36a

# IMC Experiment Workflow

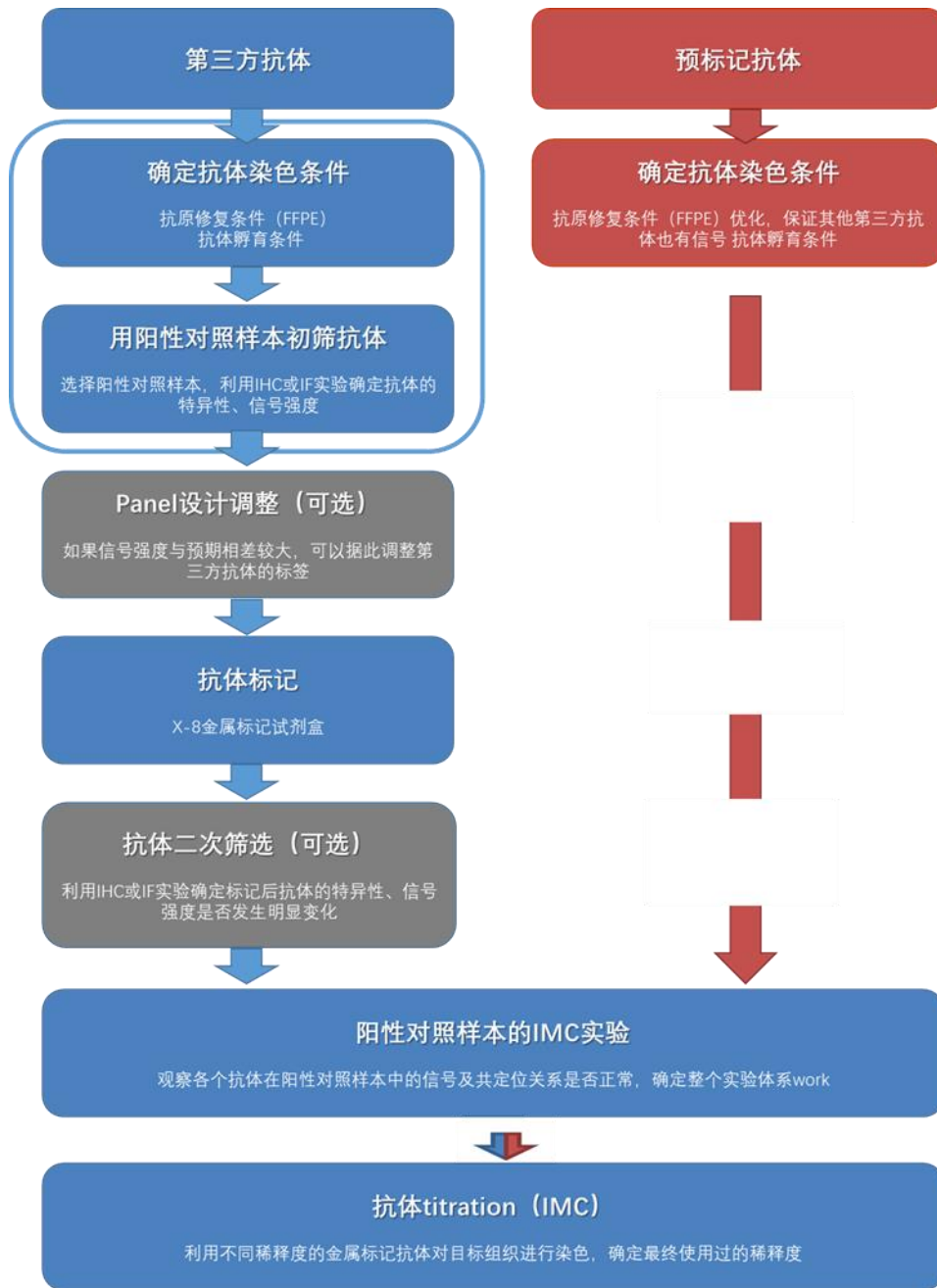
一、切片样本的准备和储存

二、实验设计

**三、Panel验证（即预实验）**

四、正式实验

五、数据分析





## A 40-Marker Panel for High Dimensional Characterization of Cancer Immune Microenvironments by Imaging Mass Cytometry

Marieke E. Ijsselstein<sup>1</sup>, Ruud van der Breggen<sup>1</sup>, Arantza Farina Sarasqueta<sup>1</sup>, Frits Koning<sup>1,2</sup> and Noel F. C. de Miranda<sup>1\*</sup>

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Multiplex immunophenotyping technologies are indispensable for a deeper understanding of biological systems. Until recently, high-dimensional cellular analyses implied the loss of tissue context as they were mostly performed in single-cell suspensions. The advent of imaging mass cytometry introduced the possibility to simultaneously detect a multitude of cellular markers in tissue sections. This technique can be applied to various tissue sources including snap-frozen and formalin-fixed, paraffin-embedded (FFPE) tissues. However, a number of methodological challenges must be overcome when developing large antibody panels in order to preserve signal intensity and specificity of antigen detection. We report the development of a 40-marker panel for imaging mass cytometry on FFPE tissues with a particular focus on the study of cancer immune microenvironments. It comprises a variety of immune cell markers including lineage and activation markers as well as surrogates of cancer cell states and tissue-specific markers (e.g., stroma, epithelium, vessels) for cellular contextualization within the tissue. Importantly, we developed an optimized workflow for maximum antibody performance by separating antibodies into two distinct incubation steps, at different temperatures and incubation times, shown to significantly improve immunodetection. Furthermore, we provide insight into the antibody validation process and discuss why some antibodies and/or cellular markers are not compatible with the technique. This work is aimed at supporting the implementation of imaging mass cytometry in other laboratories by describing methodological procedures in detail. Furthermore, the panel described here is an excellent immune monitoring tool that can be readily applied in the context of cancer research.

**Keywords:** imaging mass cytometry, cancer microenvironment, immunophenotyping, CyTOF, cancer immunity, immunotherapy

### INTRODUCTION

Technologies that support the high dimensional analysis of biological systems are essential in scientific research and have become increasingly relevant in clinical contexts. For instance, the advent of T cell checkpoint blockade therapies for cancer treatment has revitalized the field of cancer immunotherapy but also introduced an urgent need for the discovery of biomarkers that

#### OPEN ACCESS

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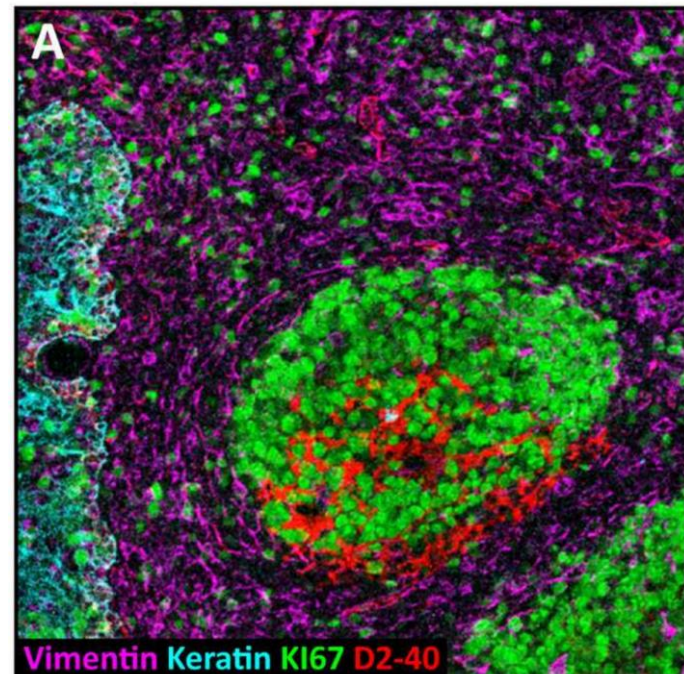
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# IMC Experiment Workflow

一、切片样本的准备和储存

二、实验设计

三、Panel验证（即预实验）

**四、正式实验**

五、数据分析



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**五、数据分析**

# A Hyperion analysis workflow

Show and Export  
image files in various  
formats



**MCD Viewer 1.0**

Single cell  
segmentation



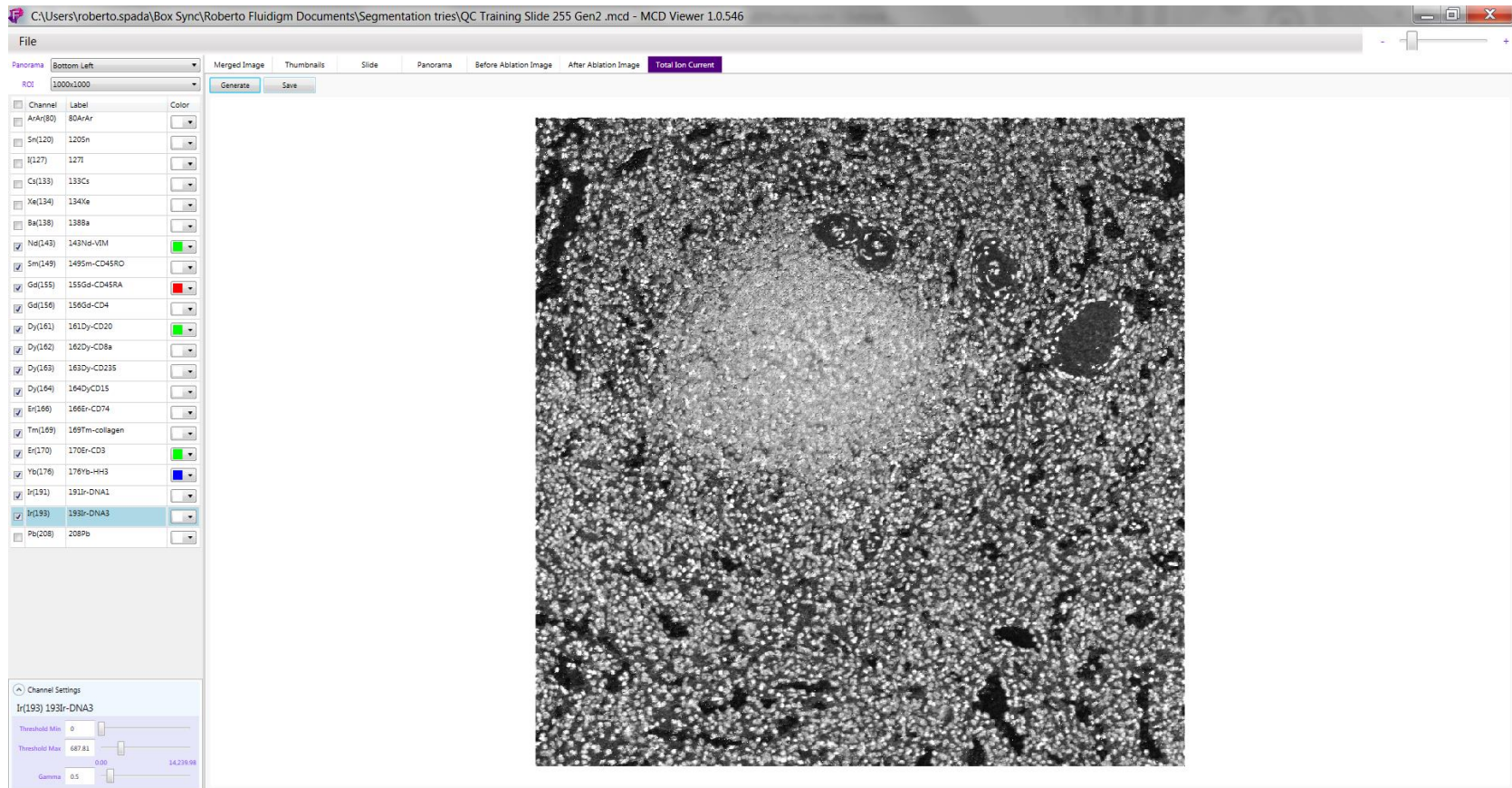
Perform higher-  
order analysis



(and other software or tool boxes)

# Step 1: Preliminary View and File Conversion

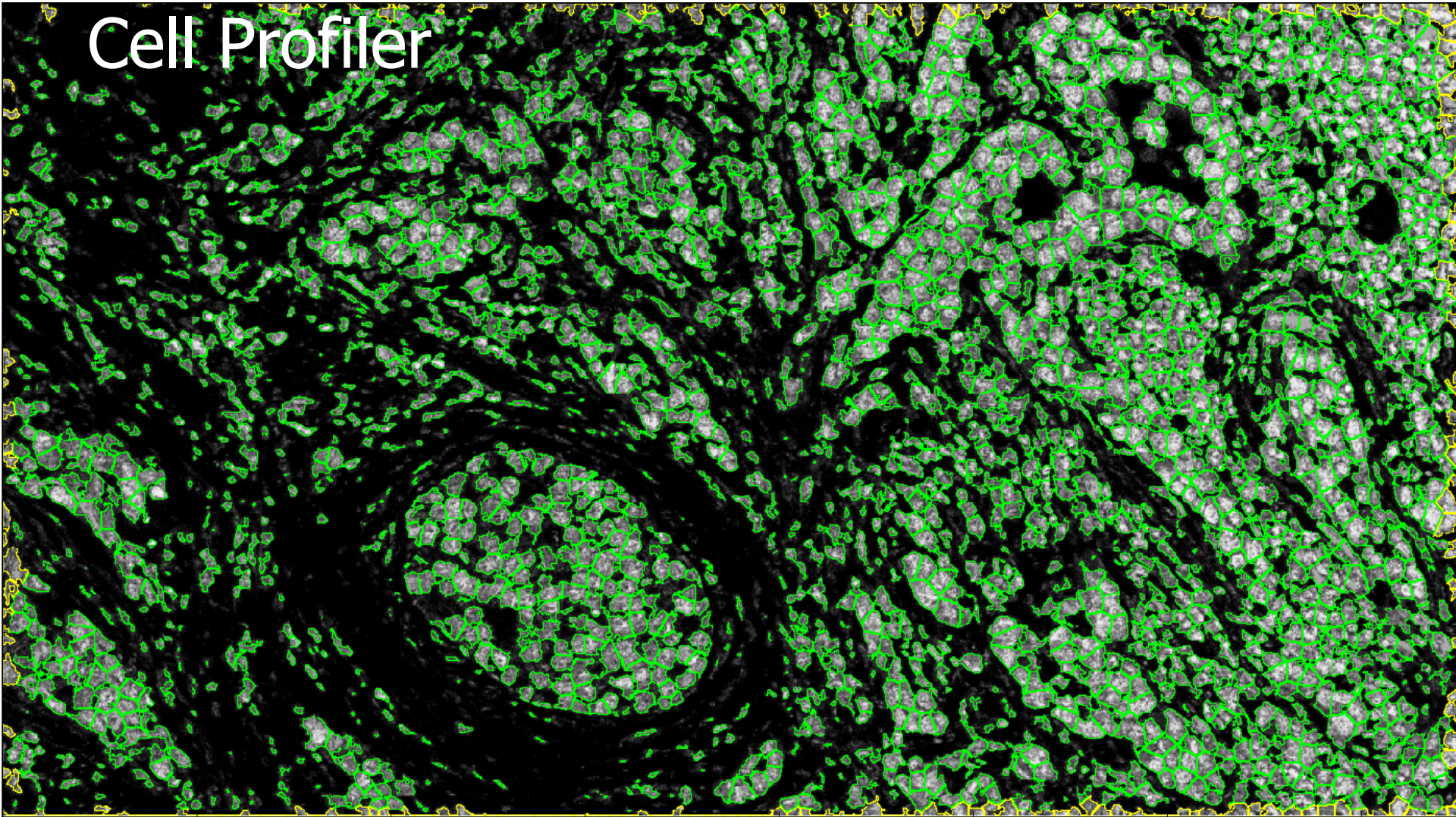
## Fluidigm MCD Viewer



Width: 1001, Height: 1000

# Step 2: Cell Segmentation

Cell Profiler

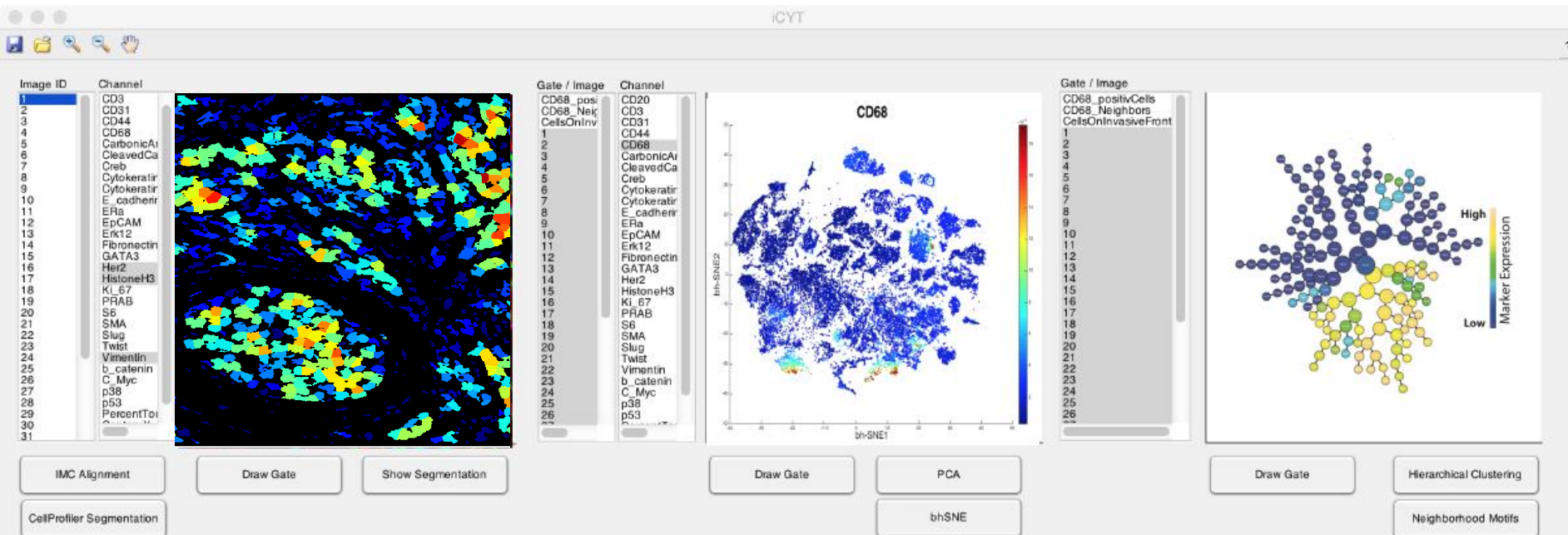


# Step 3: Perform higher-order analysis

## histoCAT Toolbox (Univ. Zurich)

Visualization

Data analysis



Data processing  
Spatial gating

Subpopulations  
Networks

Microenvironment

Clinical/patient data

**Simplify the complex  
quest to understand and  
apply biology.**



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