# VALIDATION OF IMMUNOHISTOCHEMICAL STAINING FOR NKP46 ANTIGEN, THE EFFECT OF ANTIGEN RETRIEVAL CONDITIONS

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# Research Question

Natural Killer cells (NK cells) are part of the immune system involved in cancer immunotherapy. Our interests are the validation and optimization for specificity and strength of the NKp46 antibody, a specific marker of NK cells. Staining Natural Killer cells (NK cells) specifically within tumor micro-environment (TME) can help in developing optimal processes to fluorescently tag NK cells *in situ*, providing detailed imaging to be used for analyzing spatial arrangements within TME tissue sections by the use of the Codex instrument.

- Validation of NKp46 antibody (specific for NK cells)
- Optimization through varied heat-induced epitope retrieval (HIER) conditions
- Comparison of pH9 to pH6 (standard conditions compared to CODEX protocol/specifications, respectively)

# Background

- Tumor Microenvironment (TME)
  - spatial arrangement of cellular regions composed of differing cell types based on heterogenicity
- NK cells and their immune response to tumor sites
- Heat-induced epitope retrieval (HIER)
  - paraffin embedded cancerous tissue sections
  - Temperature: > 95˚C
  - Buffer pH: 3-10

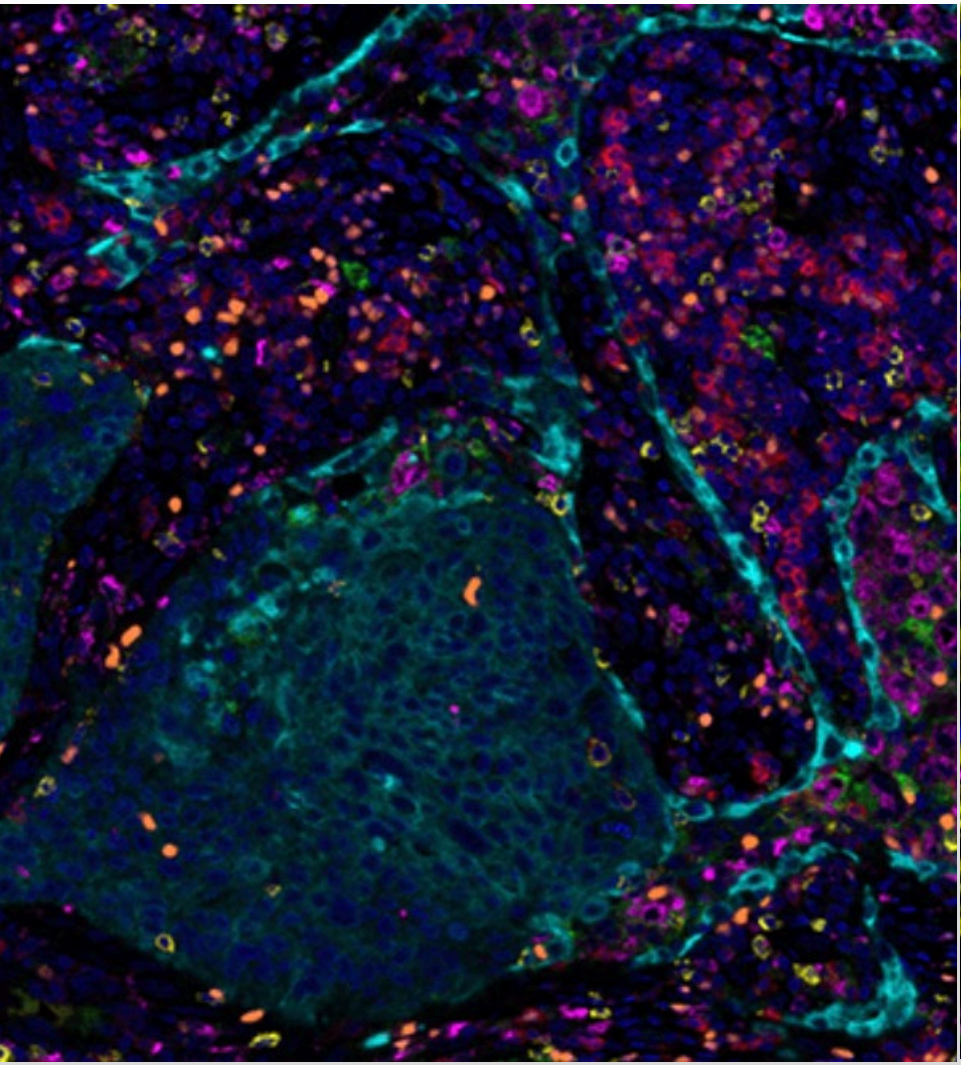
# Heat-induced epitope retrieval (HIER) conditions

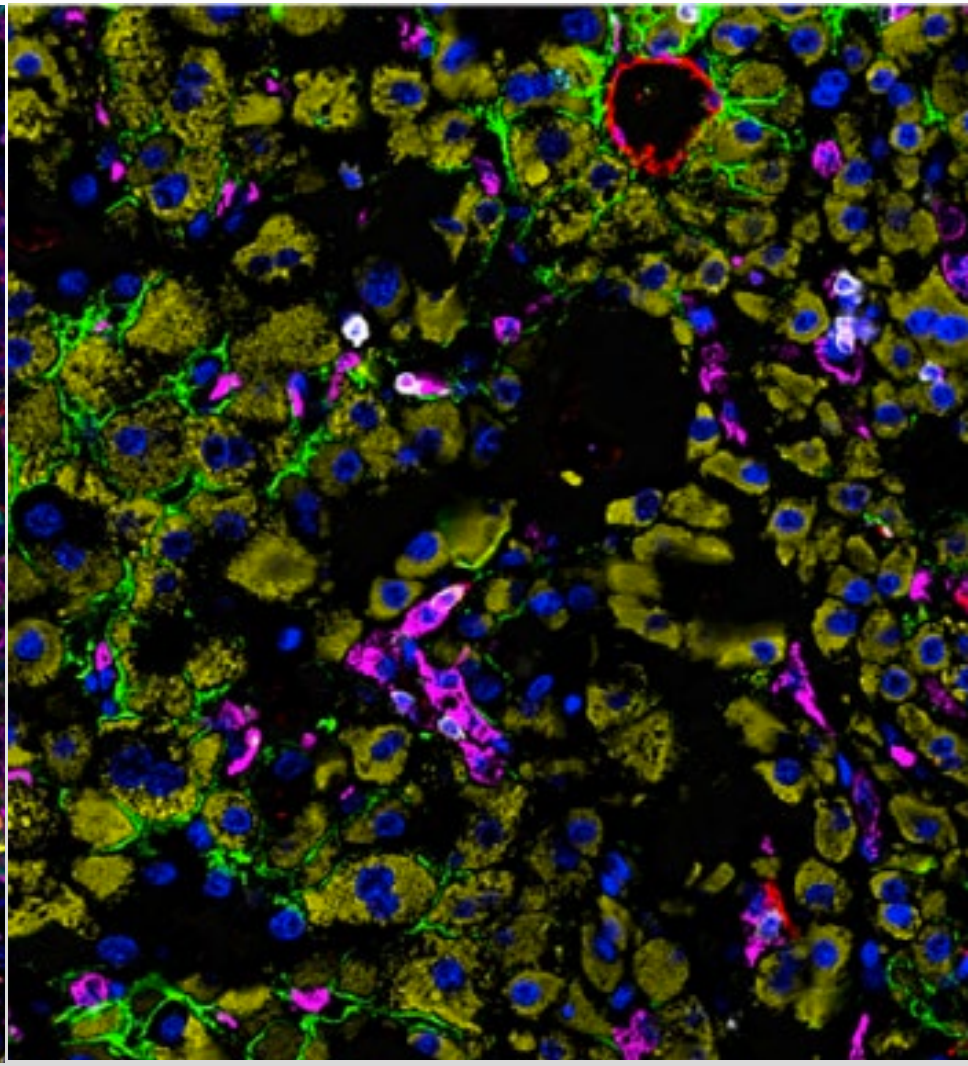
- Formalin fixation and paraffin embedding is known to alter antigenic epitopes of various antigens
- To stain for specific markers/antigens, we need to perform HIER
- Variable protocols for HIER with regards to pH, temperature, etc.
- Standard CODEX protocol within DR. G's lab is set to be pH6 at 120˚C

# Codex Instrument

- Automated fluidic system which integrates with fluorescent microscopes
  - Cyclic fluorescent tagging and image acquisition of spatial biology *in situ*
  - Provides cellular and subcellular analysis
- Method allows using up to 48 markers simultaneously within the same tissue sections
  - All antibodies must be stained using the same conditions.
  - Fluidic chemistry allows for tissue preservation and reuse
- Our codex protocol involves specific conditions of antigen retrieval

# Codex Images





https://doi.org/10.1016/J.CELL.2020.07.005

- The aim of this project is to validate and optimize staining using a novel antibody specific against NKp46 to be used in immunohistochemistry and in CODEX panel along with other multiple markers

# Materials & Methods

- Formalin fixed paraffin embedded (FFPE) Liver
  - Known to harbor NK cells
- Varied HIER conditions with NKp46 antibody
- Controls:
  - Pos: Tissue injected with purified NK cells, prepared as an FFPE tissue block
  - Neg: Similar tissue block injected with T cells
- Qualitative evaluation by hematopathologist
  - Stain intensity, tissue integrity, background quality
- Quantitative analysis
  - ANOVA (analysis of variance) for 5 top performing slides (cells/hpf)

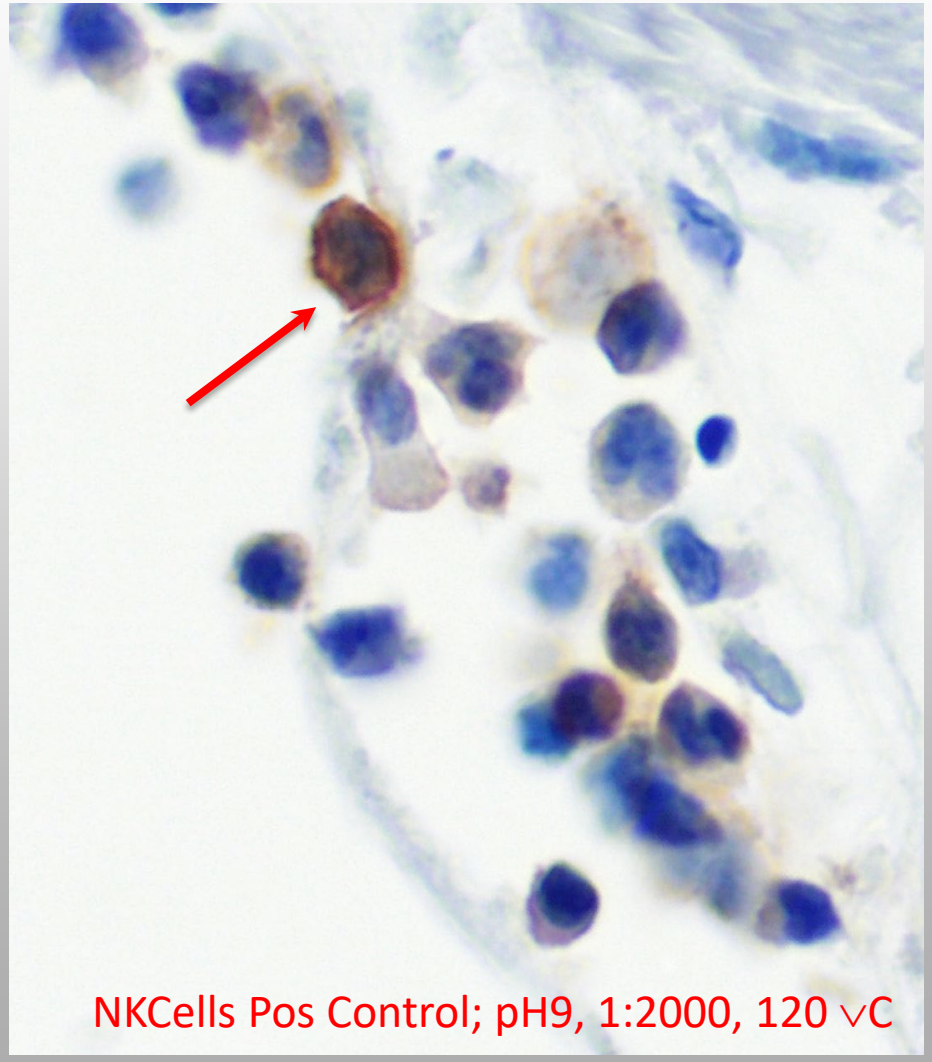
# NKp46 specific antibody

- Challenge with immunohistochemistry (IHC) - to identify antibodies that reliably and specifically stain markers of cells in FFPE tissues
- Previously tested at least 5 different antibody clones against NKp46
  - None offering reliable staining results
- A Novel clone was developed in the laboratory of Eric Vivier
  - Property of Innate Pharma, Marseilles France
- Staining protocol provided by Innate Pharma
  - Optimal antigen retrieval with pH8
  - Comparing standard conditions (pH9) to protocol of CODEX (pH6)

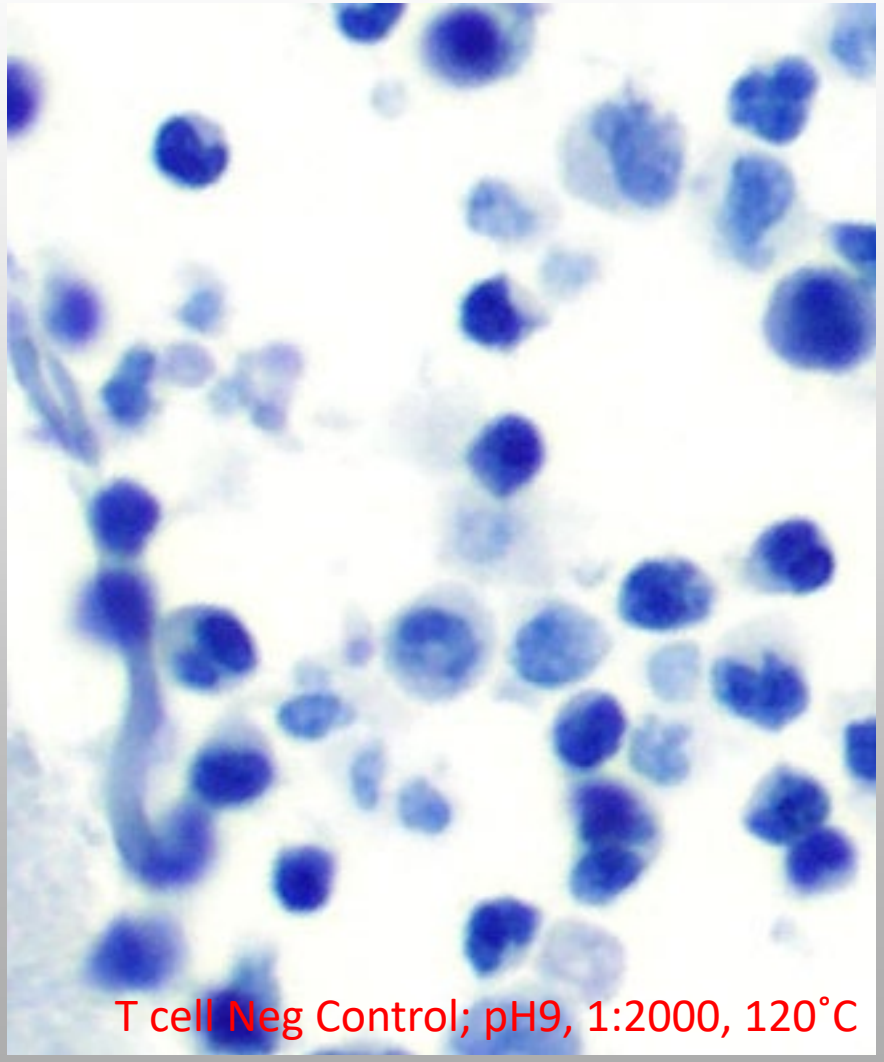
# Experimental Conditions

| Temperature | Buffer | Primary Antibody Dilutions (µg/mL) |
|---|---|---|
| 97 ˚C | pH 6 | 2.5 |
| | | 5.0 |
| | | 10.0 |
| | pH 9 | 2.5 |
| | | 5.0 |
| | | 10.0 |
| 120 ˚C | pH 6 | 2.5 |
| | | 5.0 |
| | | 10.0 |
| | pH 9 | 2.5 |
| | | 5.0 |
| | | 10.0 |

# Controls

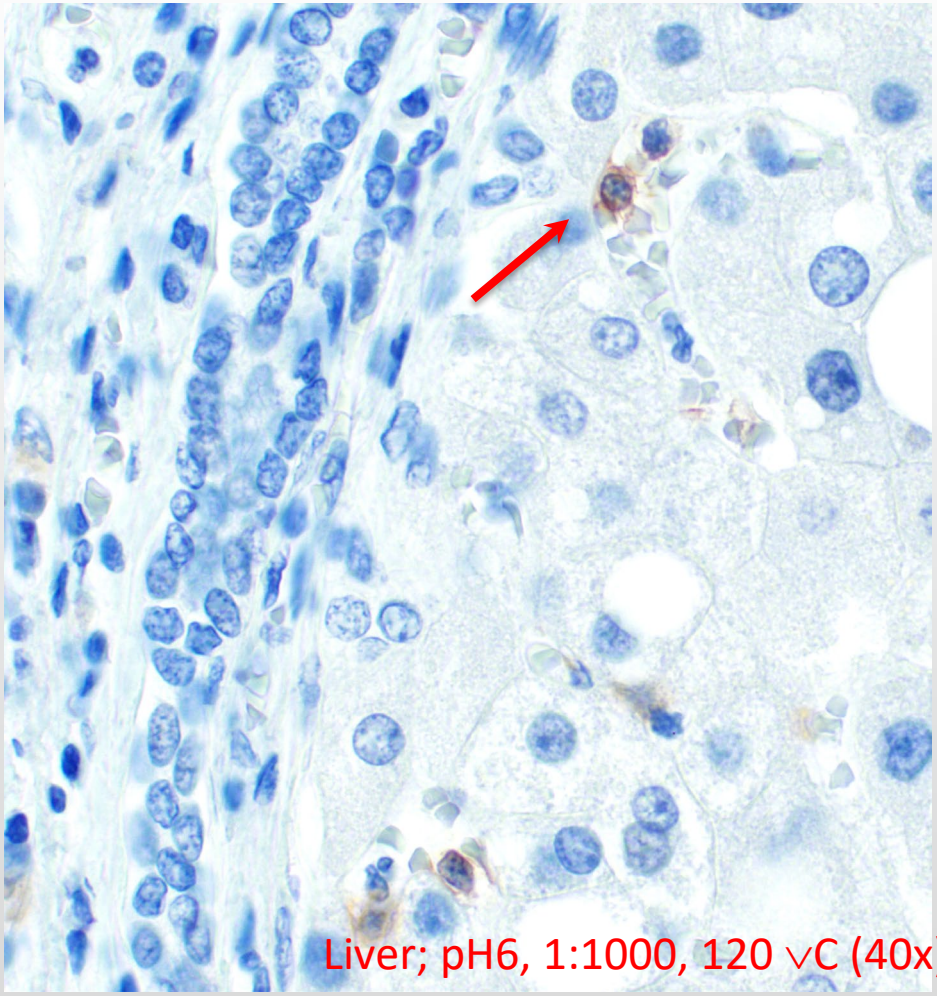


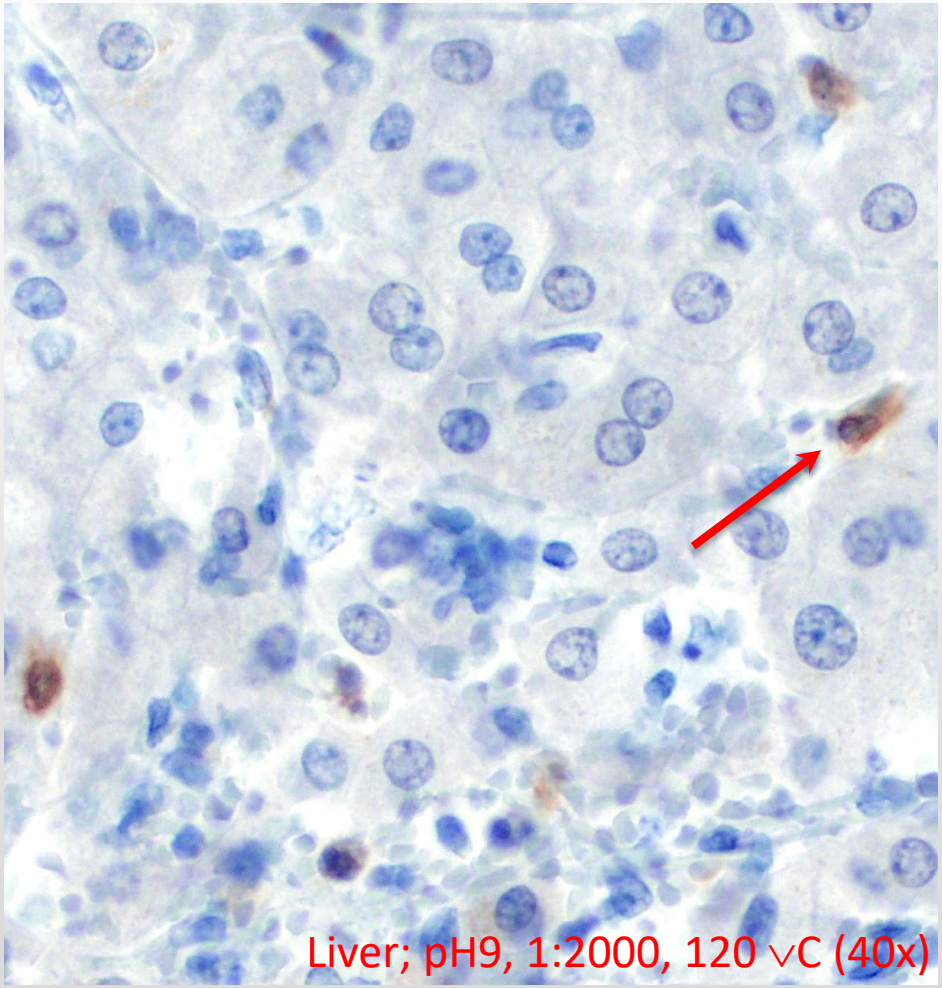
NKCells Pos Control; pH9, 1:2000, 120 ∨C



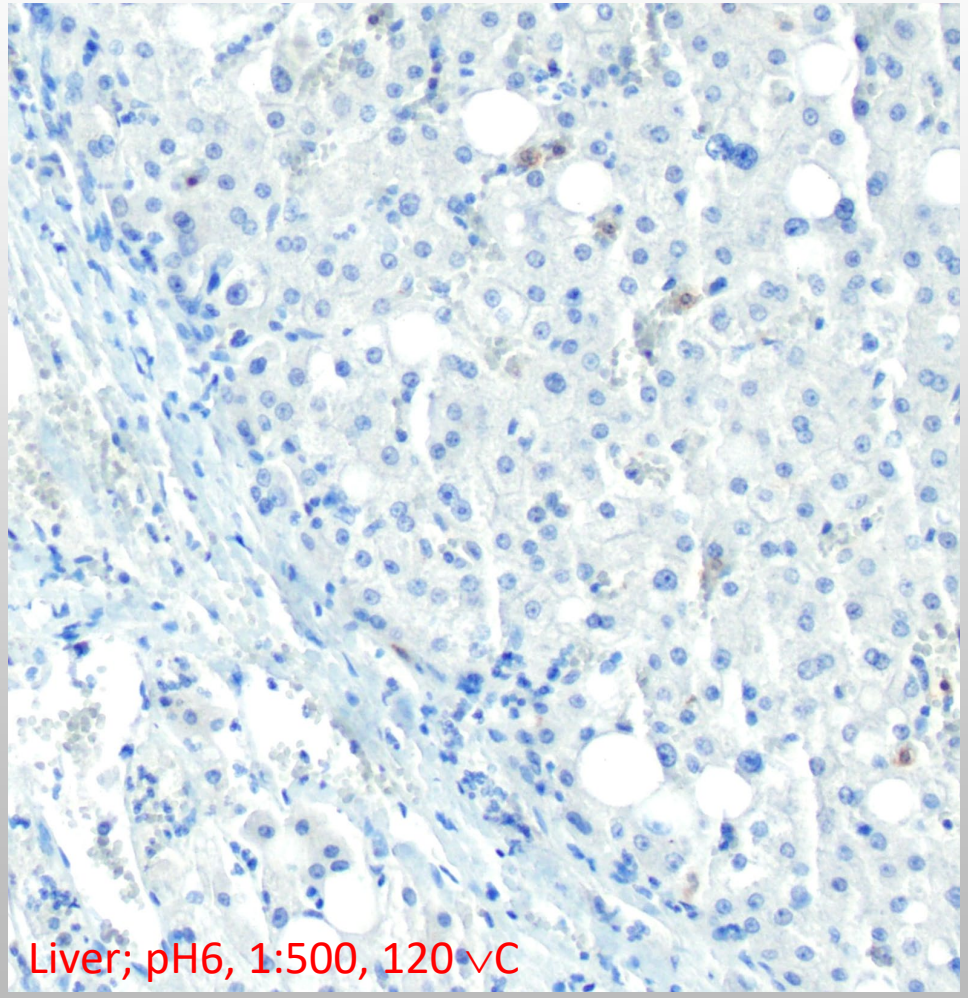
T cell Neg Control; pH9, 1:2000, 120˚C

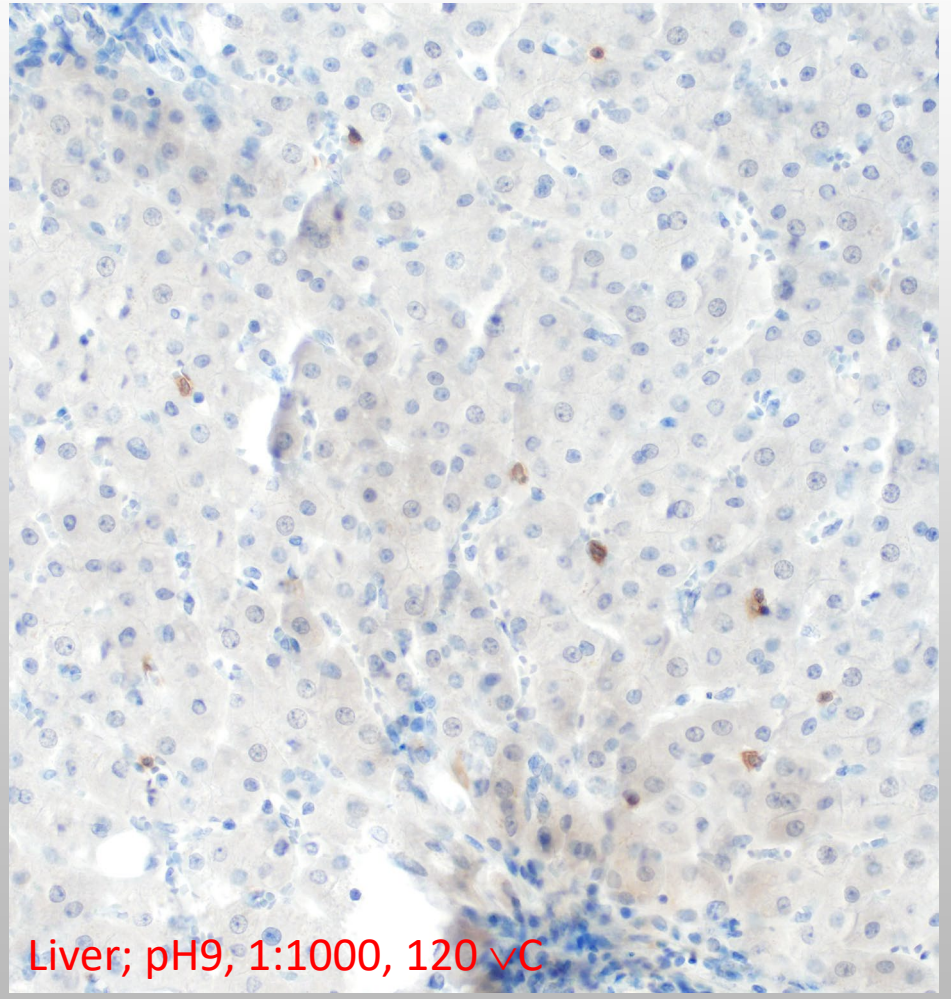
# Positive Liver IHC



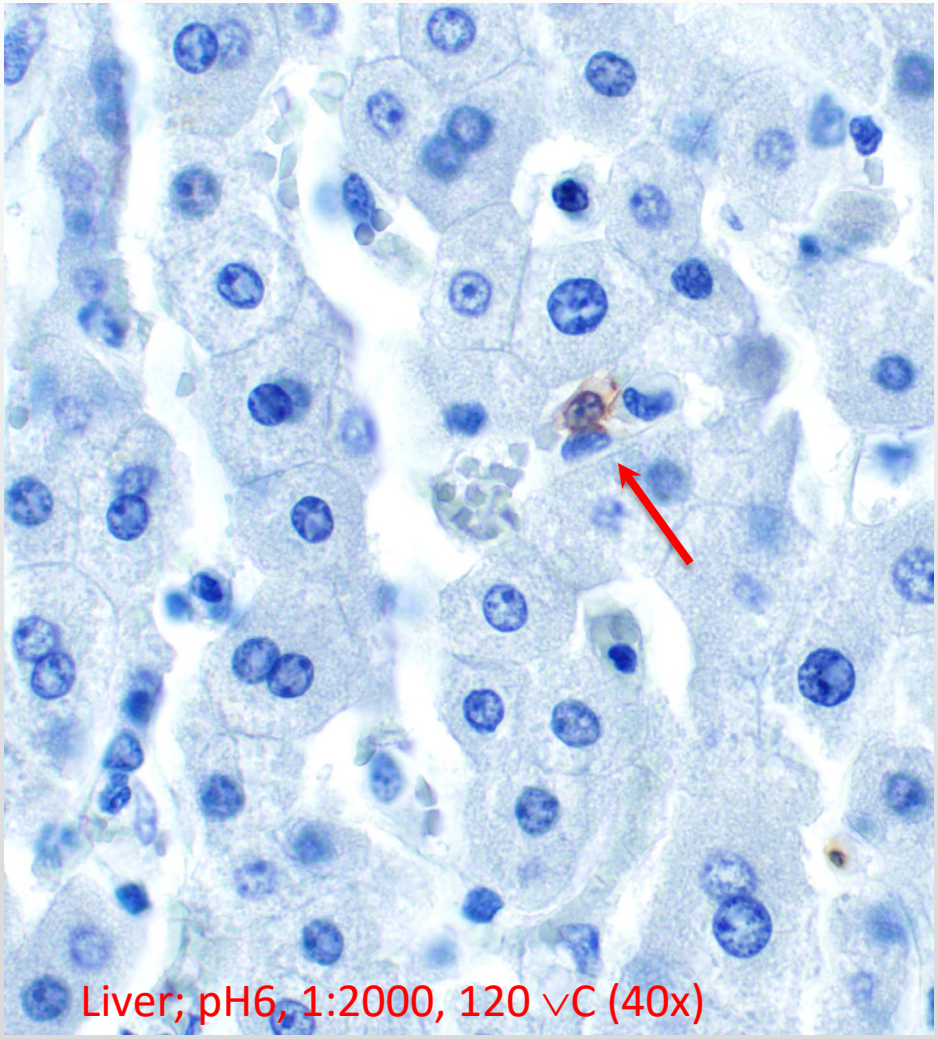



# Background Comparison

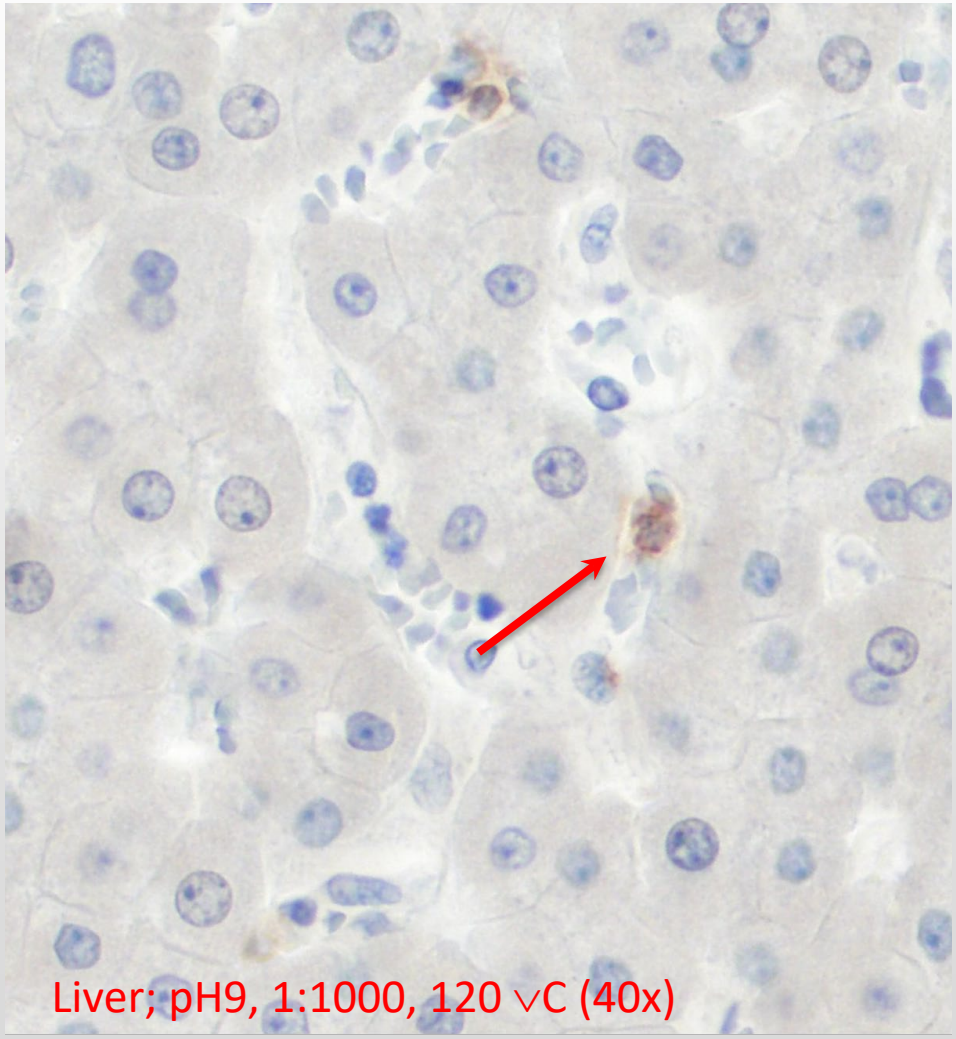

Liver; pH6, 1:500, 120 vC


Liver; pH9, 1:1000, 120 vC

# Background cont.



Liver; pH6, 1:2000, 120 ∨C (40x)



Liver; pH9, 1:1000, 120 ∨C (40x)

# Method of Evaluation of IHC

- Stain intensity
  - 0-3 scale
- Background
  - Clean
  - Slight pigmentation
  - Brown pigmentation
- Tissue integrity
  - Acceptable/unacceptable
- Semi-quantitative microscopic evaluation
  - 10-20 microscopic fields (40x) were scored
  - Results were compared using ANOVA

# Results

**Table 1: Treatment conditions and effect on stain quality**

| Temperature | Buffer | Primary Antibody Dilutions (µg/mL) | Background | Tissue integrity | Stain Intensity (0-3) | Cells/hpf (10 fields) |
|---|---|---|---|---|---|---|
| 97 ˚C | pH 6 | 2.5 | clean | acceptable | 1.5 | 0.9 |
| | | 5.0 | clean | acceptable | 2.0 | 1.6 |
| | | 10.0 | brown pigment | disintegrating | 2.0 | 1.2 |
| | pH 9 | 2.5 | clean | acceptable | 2.5 | 2.8* |
| | | 5.0 | brown pigment | acceptable | 1.5 | 2.3* |
| | | 10.0 | brown pigment | acceptable | 1.5 | 1.5 |
| 120 ˚C | pH 6 | 2.5 | clean | acceptable | 2.0 | 1.1 |
| | | 5.0 | clean | acceptable | 2.0 | 2.5* |
| | | 10.0 | clean | acceptable | 2.0 | 2.4* |
| | pH 9 | 2.5 | slight browning | acceptable | 2.5 | 3.0* |
| | | 5.0 | brown pigment | acceptable | 2.5 | 1.1 |
| | | 10.0 | brown pigment | acceptable | 2.0 | 1.7 |

*These dilutions were chosen for a 20-field cell count. n=1 for each set of conditions.

# Results

**Table 2: Average cells per field between the 5 highest performing conditions**

| *Conditions* | *Ave (SE)* |
|---|---|
| 120˚C, pH 6, 1:500 | 2.35 (1.7) |
| 120˚C, pH 6, 1:1000 | 2.85 (6.4) |
| 120˚C, pH 9, 1:2000 | 3 (2.2) |
| 90˚C, pH 9, 1:1000 | 2.15 (3.2) |
| 90˚C, pH 9, 1:2000 | 2.6 (1.3) |

p = 0.52

Conclusion:
Top five conditions give similar results without significant difference

Staining at pH6 (CODEX protocol) would give representative staining for NKp46 using this particular antibody

# Discussion / Conclusion

- 120˚C optimal temperature
  - Most reliable with stain intensity as well as least variability in qualitative outcome
- pH6 and pH9 are both acceptable
  - pH6 Adheres to CODEX protocol used in our lab
- Trade-offs when attempting higher stain intensity which increases unwanted background
  - Pigmented background can cause NK cells less to be distinguishable
- Mid to lower dilutions of NKp46 antibody demonstrated best intensity and clean background when used with the varied conditions

# Study Limitations / Next Steps

- Limited by number of tissue types (Liver)
  - Other tissue types may pose extraneous issues which remain to be seen
- Next step is to conjugate this validated/optimized NKp46 antibody with a fluorescent barcode to be used with the CODEX instrument.
- After the conjugation, the antibody will have to be validated in a CODEX run
  - Antibody may lose specificity following conjugation

# References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# Questions?