Protocol for HTT Annotation

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Part 1: ROI selection by organizers

1.1. Station configuration

- Do not use touch phones or tablets for annotation.
- Do not use touchpads for annotation. Only mouse usage is allowed.
- The evaluation set (a common set of ROIs that everyone annotates) will be used to detect outliers, including anyone who was using a suboptimal configuration that, for example, does not have enough contrast to show TILs clearly.

1.2. General remarks

- ***Visual TIL assessment guidelines***
  Please refer to section 2.1 for details and references.

- Regions-of-Interest (ROIs) are created by the organizers prior to data collection
- Each ROI size: **~500 X 500 um** (1000 x 1000 pixels @ 0.5 um/pixel)
- Number of ROIs per slide → ~10-11 ROIs
  - Acquire at 20x magnification
  - Diverse morphology from various locations within the slide

1.3. STEP 1: ROI placement

- Color indicates ROI type.
- **Note on inter-tumor variance**: We need to ensure ROIs of each of the three location categories (intra-tumoral, marginal, extra-tumoral) are representative of inter-tumor variance. Thus, of all ROIs within the tumor, we should have a representative sample of tissue with high TILs and with low or none, and everything in between. Again, for all ROIs on the margin, we should be able to show that we have a spread of all available measures (0-100/none) within our captured dataset.

- The bullet points below show the types of ROIs to pick:

- ~3 from Intra-tumoral stroma (aka tumor-associated stroma, within the boundaries of the tumor): - Green
  - Be sure to include regions with **different levels of lymphocytes (TILs)**, but you can also include **regions with high fibroblast content and few TIls**, as these may mimic TILs on poorly fixed specimens. You may also include poorly cellular regions with dense fibrous, hyalinized and collagenous tissue that contain fibroblasts and minimal amount of TILs.
  - Make sure to capture **both lymphocyte depleted and lymphocyte rich areas** within the same ROI if possible.
  - **Avoid lymphoid aggregates** (including tertiary lymphoid aggregates) as these are -strictly speaking- not considered as stromal TILs.
  - Preferable to include some tumor cells in same ROI — i.e. carcinoma cells as well as their associated stroma

- If variable TIL density within the slide, make sure to capture ROIs from multiple areas with different TIL densities.

➢ ~2 from Tumor with no intervening stroma (if possible): - Red
- Be sure to sample from: vacuolated tumor cells, dying tumor cells, regions of different densities of tumor

➢ ~2 from Invasive margin (Tumor-stroma transition): - Magenta
- If heterogeneous tumor morphology at the boundary, for example pushing versus infiltrative, sample from different tumor-stroma transitions for each

➢ ~3-4 from other regions: - Blue
- Hyalinized stroma
- Necrosis
- Normal acini/ducts
- Blood vessels
- Others at pathologist discretion.

1.4. STEP 2: sub-ROI (sROI) placement

➢ **sROI types**
- TIL-dense
- TIL-sparse

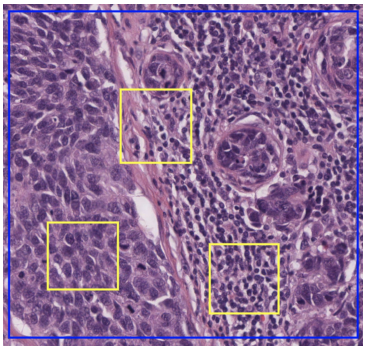
➢ Within ROI, pick sub-ROI (sROI) for TIL annotation.

➢ Each sROI is ~**125 x 125um** (256x256 pixels @ 0.5 um/pixel)

➢ In stromal regions, place 2 sROIs, one with densest lymphocytes and one with sparse lymphocytes. Even if no lymphocytes at all, still pick 2 sROIs.

➢ In tumor regions, place 1 sROI in place where intra-tumoral TIL is present (on top of cancer cell). Careful about necrotic tumor cells and other confounders. Even if there is no intra-tumoral TIL, place one sROI.

➢ Do NOT place sROIs in necrotic regions. Beware of acute or healing necrotic areas with mixed inflammatory infiltrates. Avoid those.



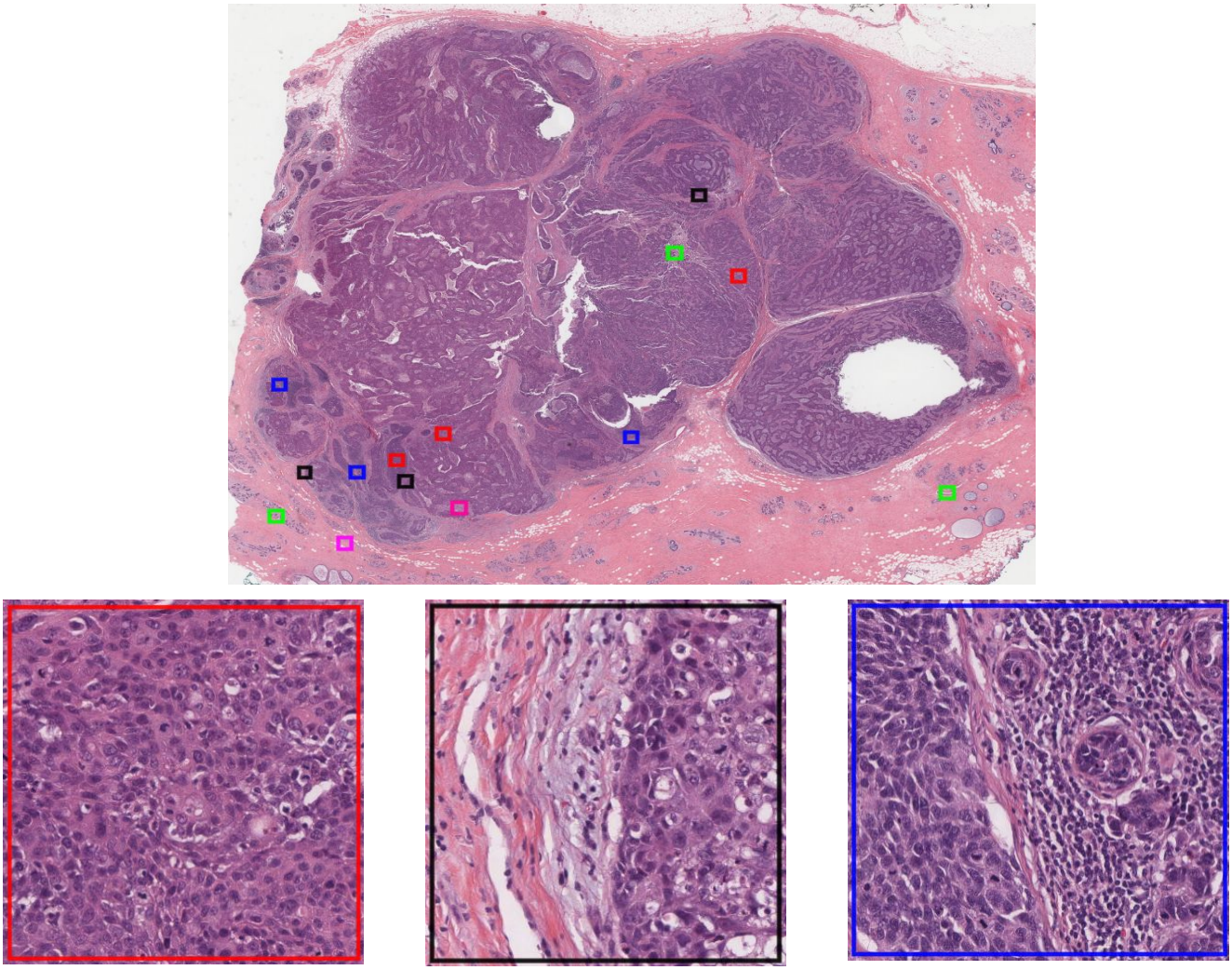


Figure 1: Sample ROIs within a slide (TCGA-A1-A0SP)

This is a NST slide (Infiltrating ductal / no specified type), the most common histological pattern. Some elements of this protocol may need modification for other histological subtypes (eg infiltrating lobular, mucinous, mixed, etc). ROIs are color coded by the type of histological pattern they capture.

Part 2: ROI/sROI annotation process

2.1. Visual TIL Assessment Guidelines

Please refer to the guidelines below before starting the annotation process.

- ➢ *Salgado, R. et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann. Oncol. 26, 259–271 (2015).*
- ➢ *Hendry, S. et al. Assessing Tumor-infiltrating Lymphocytes in Solid Tumors: A Practical Review for Pathologists and Proposal for a Standardized Method From the International Immunooncology Biomarkers Working Group: Part 1: Assessing the Host Immune Response, TILs in Invasive Breast Carcinoma and Ductal Carcinoma In Situ, Metastatic Tumor Deposits and Areas for Further Research. Adv. Anat. Pathol. 24, 235–251 (2017).*
- ➢ *For more details: see www.tilsinbreastcancer.org*

Key highlights include:

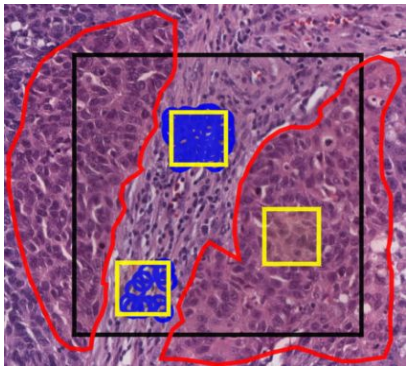
- TILs in tumor-associated stroma (stromal TILs) are prognostic and predictive for immune checkpoint inhibition in TNBC.
- Intra-epithelial TILs correlate well with stromal TILs but the guidelines support only stromal TILs due to the difficulties of assessing intra-epithelial TILs using H&E. We will, however, capture these in a few fields when annotating, to make sure algorithms can distinguish between the two TIL types.
- The invasive margin should be included in the TILs assessment.
- Tertiary lymphoid aggregates should be avoided and excluded.
- H&E is used for TILS assessment. The term “TILs”, in this context, refers to mononuclear infiltrates with lymphocyte-like morphology. Plasma cells are included in this definition, but not macrophages, eosinophils, or other immune cells.

2.2. ROI annotation process

- ➢ Participant (CROWD) goes to a URL:
  - ➢ Zooms in to the ROI on screen at 15x+

- ➢ For each ROI:
  - ➢ Annotations are color coded by class.
  - ➢ Annotate region boundaries at 20x magnification.

- ➢ **STEP 1: Annotate Region Classes (polygons)**
  - ➢ Tumor nest boundary (tumor-stroma, tumor-necrosis, …). “Tumor nest” is defined as a contiguous “region” of tumor with no intervening stroma.
  - ➢ Necrosis region boundary.
  - ➢ Do not annotate stroma — it is the “background” class by definition.



- **STEP 2: Mark TILs**
  - Drop a point on EACH and ALL TILs
  - Even if cytoplasm is visible, drop point on nucleus
  - Annotate at high (40 - 80x) magnification. Just zoom in.
  - TILs to annotate — Catch-all “TILs” class (small mononuclear infiltrates). This includes lymphocytes and plasma cells.

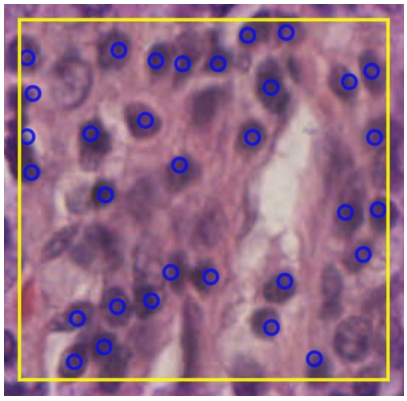
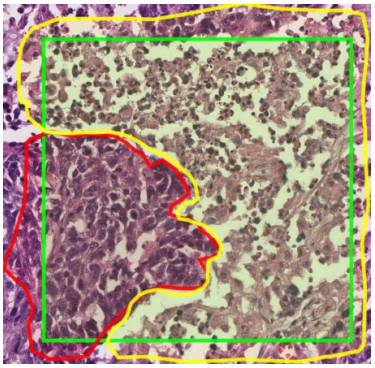
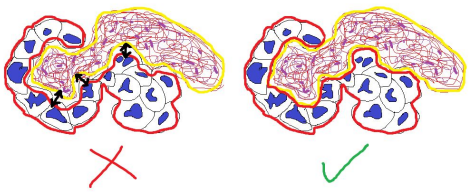
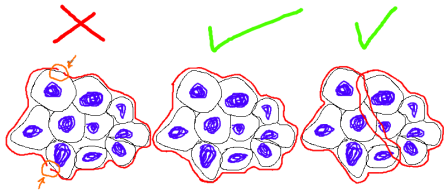
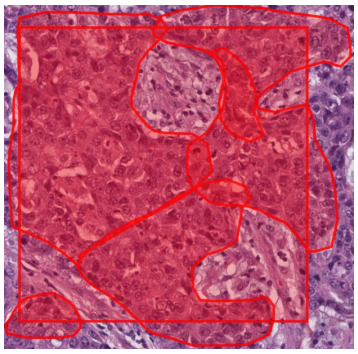
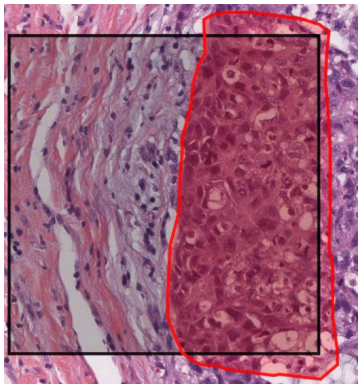
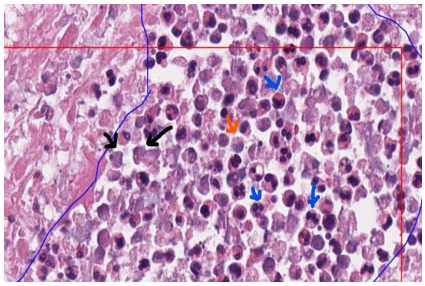
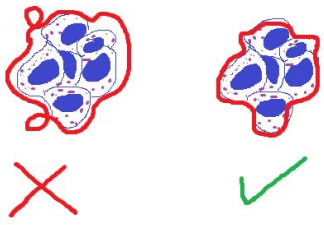
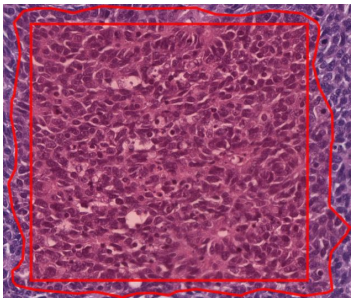
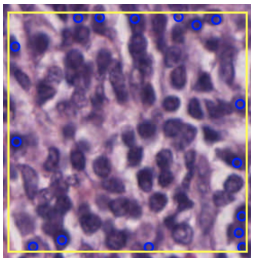


Figure 2: Sample annotated ROI at tumor-stroma transition. *Two sROIs are placed in stromal areas with high and low TIL density and one sROI is places in tumor region. Stromal TILs within the sROIs are all marked with dot annotations while Intra-tumoral TILs are marked if present.*

2.3. Additional rules + Troubleshooting

#	Rule	Rationale	Diagram
1	Mark all key regions within ROI, especially tumor and necrosis.	To control for confounders when calculating TIL score. Eg. excluding necrotic reginos from calculation.	
2	Minimize gaps between annotated regions.	Any non-annotated region is considered to be stroma by default.	

3	If region is too large to enclose in a single polygon, use overlapping polygons.	Handling annotator fatigue.	
4	Regions of the same class are allowed to overlap. Regions of different class should not overlap.		
5	If region extends beyond ROI boundary: extend polygon slightly beyond edge.	The boundary within the ROI is important, but outside boundaries are irrelevant. We want to avoid gap artifacts at ROI edge.	
6	There is a necrotic region with superimposed inflammation	Delineate necrosis region boundary and do not place and sROIs there. Inflammatory infiltrate in necrosis region is not considered in TIL scoring.	
7	Do NOT have any loops within the annotation boundaries.	Loops prevent correct rendering of boundaries to areas.	

8	If the entire ROI is tumor region, mark using an imprecise boundary external to ROI.		
9	I see an admixture of lymphocytes and plasma cells	Place a dot on nucleus (labeled by class) for lymphocytes, plasma cells, and ambiguous TILs	
10	I'm not sure if this is a TIL	Do your best and make your best professional judgement. It's understandable there is ambiguity and inter-rater variability.	
11	Nucleus extends beyond the boundary of sROI	If <u>any</u> part of the nucleus overlaps with the sROI, place a dot on the part that is inside the sROI. <u>All dots need to be inside the sROI.</u>	

2.4. Additional resources

- *Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al.* ***The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014.*** *Ann Oncol. 2015;26: 259–271.*
- *Hendry, S. et al.* ***Assessing Tumor-infiltrating Lymphocytes in Solid Tumors: A Practical Review for Pathologists and Proposal for a Standardized Method From the International Immunooncology Biomarkers Working Group: Part 1: Assessing the Host Immune Response, TILs in Invasive Breast Carcinoma and Ductal Carcinoma In Situ, Metastatic Tumor Deposits and Areas for Further Research.*** *Adv. Anat. Pathol. 24, 235–251 (2017).*
- *Savas, P., Salgado, R., Denkert, C., Sotiriou, C., Darcy, P.K., Smyth, M.J. and Loi, S., 2016.* ***Clinical relevance of host immunity in breast cancer: from TILs to the clinic.*** *Nature reviews Clinical oncology, 13(4), p.228.*
- *Amgad, M., Elfandy, H., Khallaf, H.H., Atteya, L.A., Elsebaie, M.A., Elnasr, L.S.A., Sakr, R.A., Salem, H.S., Ismail, A.F., Saad, A.M. and Ahmed, J., 2019.* ***Structured Crowdsourcing Enables Convolutional Segmentation of Histology Images.*** *Bioinformatics.*
- *Saltz, J., Gupta, R., Hou, L., Kurc, T., Singh, P., Nguyen, V., Samaras, D., Shroyer, K.R., Zhao, T., Batiste, R. and Van Arnam, J., 2018.* ***Spatial organization and molecular correlation of tumor-infiltrating lymphocytes using deep learning on pathology images.*** *Cell reports, 23(1), pp.181-193.*
- *Ørting, S., Doyle, A., van Hilten, M.H.A., Inel, O., Madan, C.R., Mavridis, P., Spiers, H. and Cheplygina, V., 2019.* ***A survey of crowdsourcing in medical image analysis.*** *arXiv preprint arXiv:1902.09159.*
- *Park et al., ESMO 2019 presentation (powerpoint can be provided).*