

# A Method to Pre-Screen Rat Mammary Gland Whole Tissue Mounts followed by RNAscope *in situ* hybridization

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## Research

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# Abstract

**Background:** RNA *in situ* hybridization is an extremely useful gene expression analysis technique that preserves the spatiotemporal nature of tissue and allows for evaluation of specific cell populations and morphologies. This technique is especially useful in evaluating expression changes in disease progression models. Tissue processing procedures used to identify pathological disease morphologies *in situ* could compromise RNA integrity and the reproducibility and quantitative accuracy of RNA *in situ* hybridization assays.

**Methods:** A combinatorial approach to pre-screen rat mammary gland tissue whole mounts for hyperplastic and malignant lesions that were not visually discernible without staining was used. This pre-screening process was followed by an RNA *in situ* hybridization analysis method known as RNAscope.

**Results:** We show that there are no differences in the quantitative nature of RNAscope assays between tissue that was immediately formalin-fixed and paraffin embedded (FFPE), which is recommended by the manufacturer, and tissue that was whole mounted and pre-screened for lesions of interest *prior* to RNAscope.

**Conclusions:** Preserving the integrity of RNA and quantitative nature of the RNAscope assay is important, as it allows unpalpable lesions to be directly identified, bypassing a need for labor-intensive serial sectioning of FFPE tissues to find lesions. This method is applicable to any epithelial-based disease progression model using whole-tissue mounts.

## Introduction:

Protein, DNA, and RNA biomarkers are important clinical diagnostic and prognostic tools [1, 2]. Gene expression profiling has highlighted an abundance of novel RNA biomarkers associated with cancer [2-4]. Expression profiling of specific RNAs using whole tissue homogenates is problematic, as signal interference from multiple cell types or structural tissue elements is introduced, and any spatial-temporal or cell-specific information is lost [2, 5]. New technologies, including single-cell sequencing and RNAscope have made single-cell RNA transcript profiling and analysis a reality. Work by Gall and Pardue in 1969 first introduced molecular hybridization using radio-labeled nucleic acid sequences *in situ* [6, 7]. Since then, RNA *in situ* hybridization (RNA ISH) has become a valuable technique that allows for gene expression analysis within a histopathological context when preserved tissue is used [5]. Commercially available RNAscope technology is an RNA ISH assay used to measure transcript levels in single cell populations in intact tissue. Target-specific signal amplification of RNAscope hybridized probes results in a visualization of individual target mRNAs as punctate dots [5, 8]. Thus, RNAscope allows for high-resolution, target-specific gene expression analysis within intact tissue.

Rodent models have been used extensively to study morphological changes during mammary gland development and disease progression [9-11]. Gross morphology of the mammary gland can be examined by whole-mounting tissue to a microscope slide and staining of intact mammary gland tissue [10, 11].

Aluminum carmine is a biological nuclear stain derived from dried female cochineal insects [12]. When applied to mammary gland whole mounts, epithelial ductal structures are easily detected within mammary tissue cleared of fat [9]. This is useful for monitoring morphological changes during mammary carcinogenesis. It is relevant to visualize rat mammary gland ductal structures for pre-clinical research purposes because the vast majority of human breast malignancies arise from ductal epithelial cells [13-15]. Rat mammary carcinogenesis serves as a model of the natural histopathological lesions and malignancies found in human breast disease. Screening tissue whole mounts is a valuable technique to visualize morphological changes occurring during carcinogenesis [13, 16].

RNAscope assays are designed and recommended to be used in recently processed formalin fixed and paraffin embedded (FFPE) or cryopreserved tissue samples. There is a pervasive problem of nucleic acid degradation when handling tissue with any ISH assay, including RNAscope. Nucleic acid quality has effects on the reproducibility and quantitative analysis of downstream assays. Protein, DNA, and RNA are better preserved, for a longer period of time in FFPE tissues compared to frozen tissues [17, 18]. Morphological features are also retained in FFPE tissues, which has made it a preservation method of choice for decades.

Immediate FFPE processing of tissue intended for RNAscope analysis is recommended to preserve the integrity of RNA. This is because the duration of fixation and pre-treatment conditions can negatively impact nucleic acid integrity [19, 20]; therefore, care must be taken to limit exposure time and harsh conditions prior to paraffin embedding. In this methods report, we outline how RNAscope can be effectively utilized following screening of mammary gland whole tissue mounts for lesions of interest. This is useful as it allows for identification and study of diseased tissue morphologies without apparent loss of RNA integrity. We show, using probes targeting genes of different expression levels, that the quantitative capacity of the RNAscope assay is retained following whole tissue mounting and processing. This is applicable to any epithelial-based disease progression model requiring pre-screening of whole tissue.

## **Protocol:**

### *Animal husbandry*

Rats were maintained in an AAALAC-approved facility on a 12 h light/dark cycle and provided LabDiet 5001 Rodent Diet (PMI® Nutrition International) and water ad libitum. Inbred Wistar Furth (WF/Hsd) females were obtained from Envigo. All animal protocols were approved by the University of Louisville Animal Care and Use Committee.

### *DMBA Administration and Mammary Gland Tissue Resection*

At 50-55 days of age 7,12-Dimethylbenz(a)anthracene (DMBA Acros Organics, CAS#: 57-97-6, 20 mg/mL in sesame oil) was given by single oral gavage (65 mg DMBA/kg body mass) to WF/Hsd (Envigo) females to induce mammary carcinogenesis. Mammary glands were resected four or twelve weeks

following DMBA administration. Briefly, one skin incision was made at the lower abdomen and extended forward, along the midline, to the neck. Four additional skin incisions were made, each extending from the midline down each limb. Skin was separated from the body cavity to reveal mammary glands present in pairs along each ventro-lateral boundary of the body. The larger mammary glands, such as the combined thoracic pectoral glands toward the anterior (Fig 1, labeled B/C glands) or individual abdominal-inguinal glands toward the posterior (Fig 1, labeled D glands) were excised for whole mounting. Mammary gland tissue was carefully trimmed away from the skin, moving as far dorsally as possible. One abdominal D mammary gland was immediately processed by formalin fixation and paraffin embedding (FFPE), while the other was whole mounted and processed as outlined below.

#### *Whole Mount Preparation:*

Immediately following tissue resection, mammary glands were stretched across an electrostatically charged slide and submerged in room temperature, 10% neutral buffered formalin overnight for fixation. Slides were removed from formalin and placed into an acetone bath at room temperature overnight to defat. Overnight may not be enough time to entirely remove all fat; however, overnight is sufficient and allows for sooner and more visible staining. Mammary glands will become translucent and may require up to 3 days in acetone to remove fat, depending on the thickness of the mammary gland.

Following defatting of the mammary glands, slides were submerged in aluminum carmine staining solution. Aluminum carmine solution was prepared by dissolving 1g of carmine alum and 2.5g of aluminum potassium sulfate in 500 mL of RNase-free water. Boiling 20 minutes was used to completely dissolve carmine into solution. Carmine solution was refrigerated for storage and reused after filtering to remove buildup of oil remaining in mammary glands. Staining may take 1-2 days depending on mammary gland thickness. Glands were considered fully stained when white tissue was no longer visible through the back of the slide. Whole mounted fixed tissue not used for the purposes of RNAscope analysis can be stored long-term submerged in mineral oil.

#### *Mammary gland tissue processing, embedding, and sectioning:*

To evaluate morphological structures and lesions within carmine stained mammary gland tissue whole mounts, such as hyperplasia and ductal carcinoma *in situ* (DCIS) (Fig 2), regions of interest were excised from whole-mounted glands using a surgical scalpel. Excised tissue was placed in histology cassettes and processed using a standard tissue processor. To properly embed tissue in paraffin blocks, tissues were oriented such that the flat side, originally against the glass microscope slide, faced the bottom of the paraffin mold. Paraffin embedded tissues were stored at 4°C until ready to section. Messenger RNA is stable in paraffin blocks for effective use in RNAscope for up to 1 year [21].

Sectioning of FFPE blocks was done no more than one day *prior* to a planned RNAscope assay, as mRNA remains more stable within paraffin blocks than tissue sections [21]. Prior to sectioning, paraffin blocks were placed at -20°C for a minimum of 30 minutes to improve sectioning and reduce wrinkling of tissue sections. Tissues were cut into 4 mm sections and placed onto superfrost plus microscope slides. Other

slide types, suitable for RNA *in situ* hybridization assays, may also be used. Slides were dried fully at room temperature overnight. Depending on tissue size, it was possible to fit up to three sections per slide. This served to analyze multiple genes or controls on a single slide in an RNAscope assay.

#### *Hematoxylin and Eosin (H&E) staining of sectioned mammary glands:*

An H&E stain on sectioned tissues was used to assess tissue sample quality and to confirm the lesion of interest was present in the section. Slides were deparaffinized and rehydrated using 3 separate xylene washes followed by single washes each in 100% ethanol, 90% ethanol, 80% ethanol, and DI water. Tissues were stained with hematoxylin for 3 minutes, rinsed under running DI water for 3 minutes, soaked in bluing reagent for less than a minute, and again rinsed with DI water. Slides were dipped a few times in 100% ethanol to eliminate water before staining in eosin-Y for 30-45 seconds. Tissue was then dehydrated using washes of 90% ethanol, 100% ethanol, and 3 separate xylene washes. Samples were air dried and coverslipped using permount or similar mounting media

#### *RNAscope Assay*

In this report, RNAscope assays were performed on pairs of D mammary glands, one immediately processed by FFPE and the other whole mounted, defatted, carmine stained, and processed and paraffin embedded later. The RNAscope 2.5 HD Detection Reagent-RED kit (Advanced Cell Diagnostics #322360) was used according to manufacturer's protocol except for modifications to minimize harsh pre-hybridization treatment conditions on fragile mammary tissue. Slides were deparaffinized in two xylene, followed by two 100% ethanol washes. A hydrophobic barrier was drawn around tissue sections with an ImmEdge pen. Sections were blocked with H<sub>2</sub>O<sub>2</sub> for 10 minutes at room temperature. Slides were submerged in target retrieval solution at a temperature just below boiling for 10 minutes, rather than the manufacturer recommended boiling for 15 minutes. Protease plus solution was added to slides and incubated in HybEZ oven (ACD Bio) at 40°C for 10 minutes, rather than the manufacturer recommended 30 minutes. Target probe was added and allowed to hybridize for 2 hours at 40°C in the HybEZ oven. Serial steps to amplify signal involved the following solutions and conditions: Amp1 for 30 minutes at 40°C, Amp2 for 15 minutes at 40°C, Amp3 for 30 minutes at 40°C, Amp4 for 15 minutes at 40°C, Amp5 for 30 minutes at room temp, Amp6 for 15 minutes at room temp, and detection reagent for 10 minutes at room temperature. Slides were washed in wash buffer between each probe hybridization and amplification step. Slides were counterstained with 1:1 Gill's hematoxylin and water, allowed to air dry, and coverslipped with mounting media. Slides were imaged on an Aperio ImageScope CS2.

#### *Expression Quantification and Statistics:*

Representative images were selected from n=6 mammary carcinoma and n=6 non-diseased mammary tissues that were either immediately FFPE processed or whole mounted prior to FFPE processing. Mean probe *per cell* was quantified for *DapB*, *Mier3*, and *Polr2a* expression using the HALO-ISH Quantification software from Indica Labs. Two independent DMBA groups were used to provide mammary gland or mammary carcinoma tissue, each tissue type was analyzed separately by Two-Way ANOVA. Independent

variables to compare mammary gland and carcinoma expression levels were processing method and RNAscope probe. Probe per cell was the dependent variable. Tukey's *post hoc* test was used protect for multiple comparisons. Statistical analysis was performed using GraphPad Prism version 7.00 for Windows, GraphPad Software.

## Results & Discussion:

Mammary gland whole mounts, are used to detect various stages of pathological mammary gland lesions, including pre-malignant hyperplasias, pre-invasive ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma (IDC). Standard histopathologic approaches, such as hematoxylin and eosin (H&E)-staining of FFPE tissue sections, might miss morphological abnormalities not captured within evaluated tissue serial sections [11]. Whereas, pre-screening mammary gland whole-tissue mounts for abnormal morphologies allows for direct identification of lesions and hyperplastic regions. These regions can be excised and further processed separately for analysis by tissue sectioning.

Here, aluminum carmine stained whole tissue mount screening was used to detect mammary ductal epithelium morphological changes that occur during mammary cancer progression. Mammary ductal structures, interspersed within stromal tissue, are visible after staining with aluminum carmine [22]. Early hyperplastic proliferative lesions appeared, as expected, in aluminum carmine stained mammary gland whole mounts (Fig 2)[9, 10, 22]. These lesions were extended, thickened, and amplified ductal branches. Ductal carcinoma *in situ* (DCIS), an intraductal proliferation of epithelial cells, (Fig 3)[13] appeared as darkened structures within whole mounts (Fig 2). This intense staining is due to a dense concentration of epithelial cells present in these lesions. The ability to detect and study epithelial-based diseased tissue morphologies within mammary glands following aluminum carmine staining is crucial since breast cancer predominantly arises from luminal epithelial cells lining ducts [13, 14]. Invasive ductal carcinoma (IDC) presented in tissue sections as migratory epithelial cells penetrating ductal walls and invading stroma (Fig 3) [13, 15]. These carcinomas appear as palpable mammary tumors that are visible without aluminum carmine staining.

Whole mount tissue processing, followed by RNAscope, was successfully used in this study to produce RNAscope results that were similar to results from samples directly processed by FFPE. Therefore, an exposure to additional reagents and time, which was required for whole tissue mounts processing *prior* to paraffin embedding, did not have a negative effect on mRNA quality or abundance. *Four-hydroxy-tetrahydrodipicolinate reductase (DapB)* is a bacterial gene that is not present in properly handled mammalian samples. No positive staining for DapB was observed using RNAscope in samples used in this study (Fig 3). This indicated no bacterial contamination occurred while processing whole mounts. To test the sensitivity of RNAscope following whole tissue mounting, we targeted the *mesoderm induction early response family member 3 (Mier3)* gene, a low to moderately expressed gene transcript in rat mammary and female breast tissue, with average expression of 11 transcripts per million [23]. For comparison, we probed for *RNA Polymerase II Subunit A (Polr2A)*, a highly expressed gene with an average of 79 transcripts per million in human breast tissue [23]. RNAscope assays probing for *Polr2a*

and *Mier3* reflected these expected expression differences (Fig 3). Results observed for *Polr2a* were densely packed red puncta within mammary ducts, while a *Mier3* probe resulted in more scattered red puncta. There was no discernable difference in puncta abundance between RNAscope assays performed on aluminum carmine stained mammary gland whole mounts and mammary glands that were formalin fixed and paraffin embedded immediately following tissue resection (Fig 4). This lack of difference between tissue treatment methods, and an expected difference in transcript abundance between independent gene target probes, indicated that a pre-screening process did not negatively impact mRNA stability.

To verify that similar quantitative puncta density results were obtained from different tissue processing methods, RNAscope images were quantified using HALO-ISH software (Indica Labs) (Fig 5). Non-diseased mammary ducts displayed means  $\pm$  standard deviation (SD) for *DapB* probe *per* mammary epithelial cell of  $0.28 \pm 0.26$  by immediate FFPE and  $0.35 \pm 0.19$  by tissue whole mounting. An RNAscope probe for *Mier3* resulted in means  $\pm$  SD per cell of  $2.34 \pm 0.46$  in immediate FFPE and  $2.32 \pm 0.14$  in tissue processed from whole-mounted mammary glands. The *Polr2a* probe yielded means  $\pm$  SD *per* mammary epithelial cell of  $7.55 \pm 1.22$  in immediately processed FFPE tissue and  $7.21 \pm 1.22$  in whole mounted tissue. A two-way ANOVA indicated no statistically significant difference in quantities from tissues that were immediately FFPE processed according to the manufacturer's directions, or tissues that were whole mounted prior to FFPE processing. There was a statistically significant difference between gene probes, as expected ( $p < 0.0001$ ). This confirmed anticipated results of no to low *DapB* expression, moderate *Mier3* expression in both tissue processing methods, and high *Polr2a* expression, which indicates the quantitative sensitivity of RNAscope assays was retained in whole mounted tissue. Similar results between tissue processing methods were obtained when RNAscope assays from mammary carcinomas were quantified. Means  $\pm$  SD of the *DapB* probe *per* carcinoma cell were  $0.15 \pm 0.17$  in immediate FFPE tumors and  $0.23 \pm 0.20$  in whole mounted tissue containing a tumor. A *Mier3* probe displayed means  $\pm$  SD per cell of  $4.45 \pm 0.64$  in immediate FFPE tumor tissue and  $4.30 \pm 0.59$  in whole mounted tumor tissue. The *Polr2a* probe resulted in means  $\pm$  SD per cell of  $12.27 \pm 1.65$  in immediate FFPE processed carcinomas and  $11.52 \pm 1.90$  in whole mounted carcinoma tissue. A two-way ANOVA was not statistically significant for tissue processing method, but was significant for assay probe ( $p < 0.0001$ ). These results validate that RNAscope is a robust and appropriate procedure for quantitative analysis of gene expression in whole mounted tissue followed by FFPE.

## Conclusion:

RNAscope can be successfully used on FFPE tissue that was initially processed as whole mounted tissue. There was no loss in qualitative or quantitative values of RNAscope. This allows for pre-screening of potential lesions or morphological tissue changes that are only detectable with tissue staining. Crucially, methods outlined in this report provide a way to classify and separately process stages of disease progression while retaining the target specificity and robust quantitative nature of the RNAscope assay.

## Abbreviations

FFPE: formalin-fixed and paraffin embedded

RNA ISH: RNA *in situ* hybridization

DMBA: 7,12-Dimethylbenz(a)anthracene

WF: Wistar Furth

DCIS: ductal carcinoma *in situ*

IDC: invasive ductal carcinoma

H&E: Hematoxylin and Eosin

DapB: Four-hydroxy-tetrahydrodipicolinate reductase

Mier3: mesoderm induction early response family member 3

Polr2A: RNA Polymerase II Subunit A

## Declarations:

*Ethics approval and consent to participate:*

All animal protocols were approved by the University of Louisville Animal Care and Use Committee.

*Consent for publication:*

Not applicable

*Availability of data and materials:*

The datasets generated and/or analyzed during this study are not publicly available because they are saved on private servers, but may be available from the corresponding author upon request.

*Competing interests:*

The authors declare no competing interests

*Funding:*

The University of Louisville Integrated Programs in Biomedical Sciences (to ELD) and the Department of Biochemistry and Molecular Genetics

*Authors contributions:*

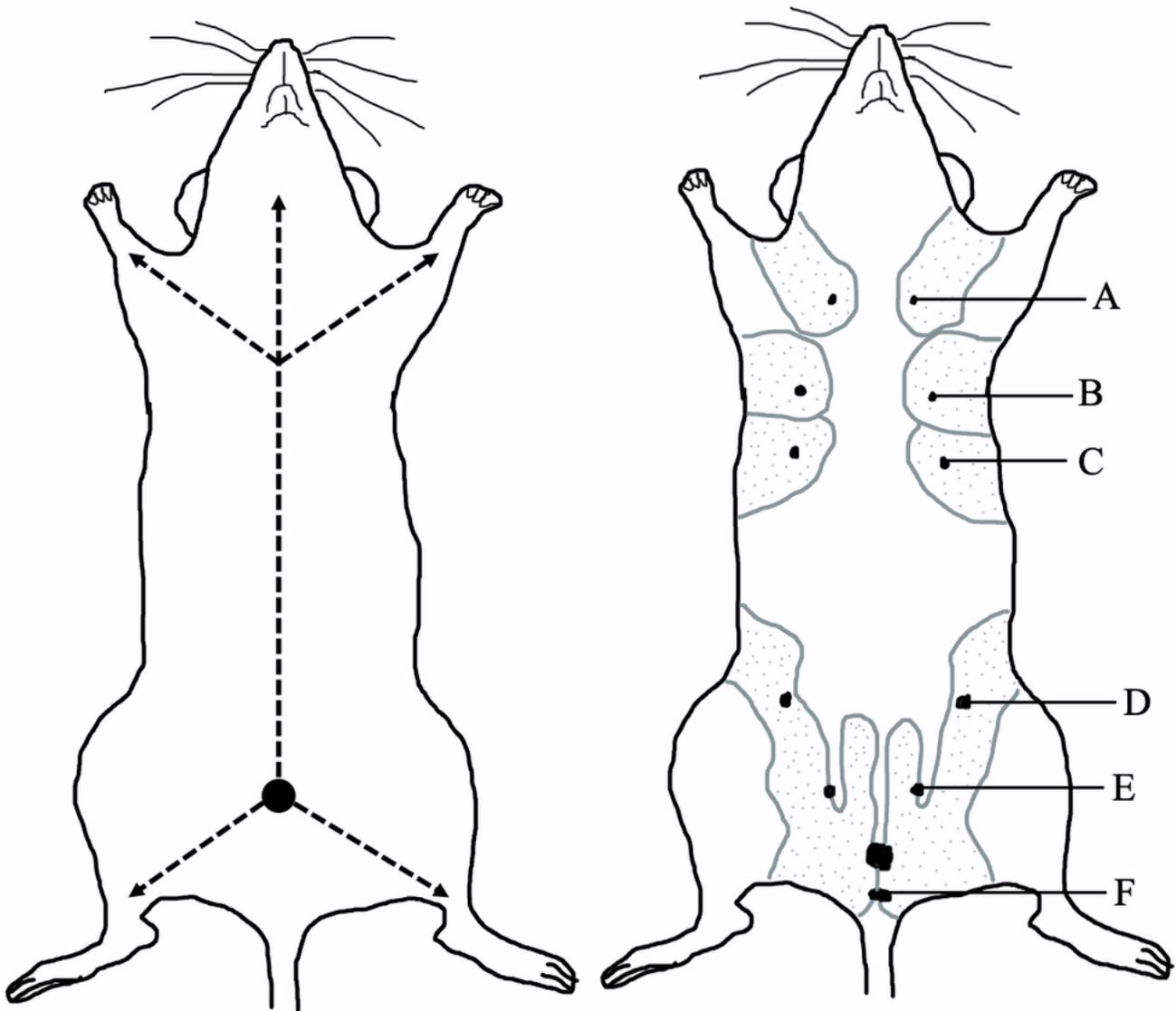
ELD administered DMBA, performed tissue dissection and whole mount preparation, tissue sectioning and staining, RNAscope assays, and image quantification. MGS assisted in tissue morphology and disease classification. ELD and DJS did experimental design, statistical analysis, figure preparation, and writing of the manuscript.

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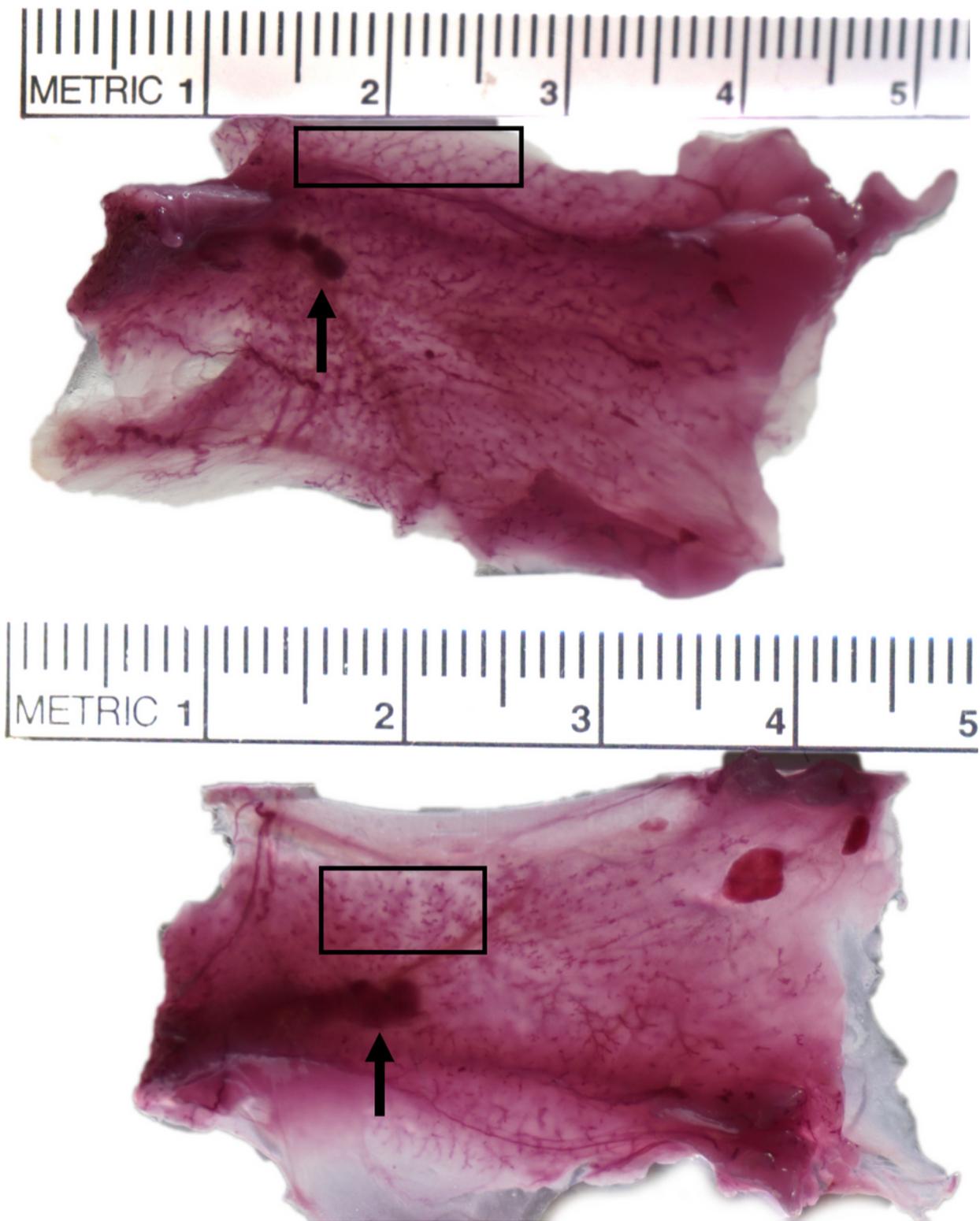
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## Figures



**Figure 1**

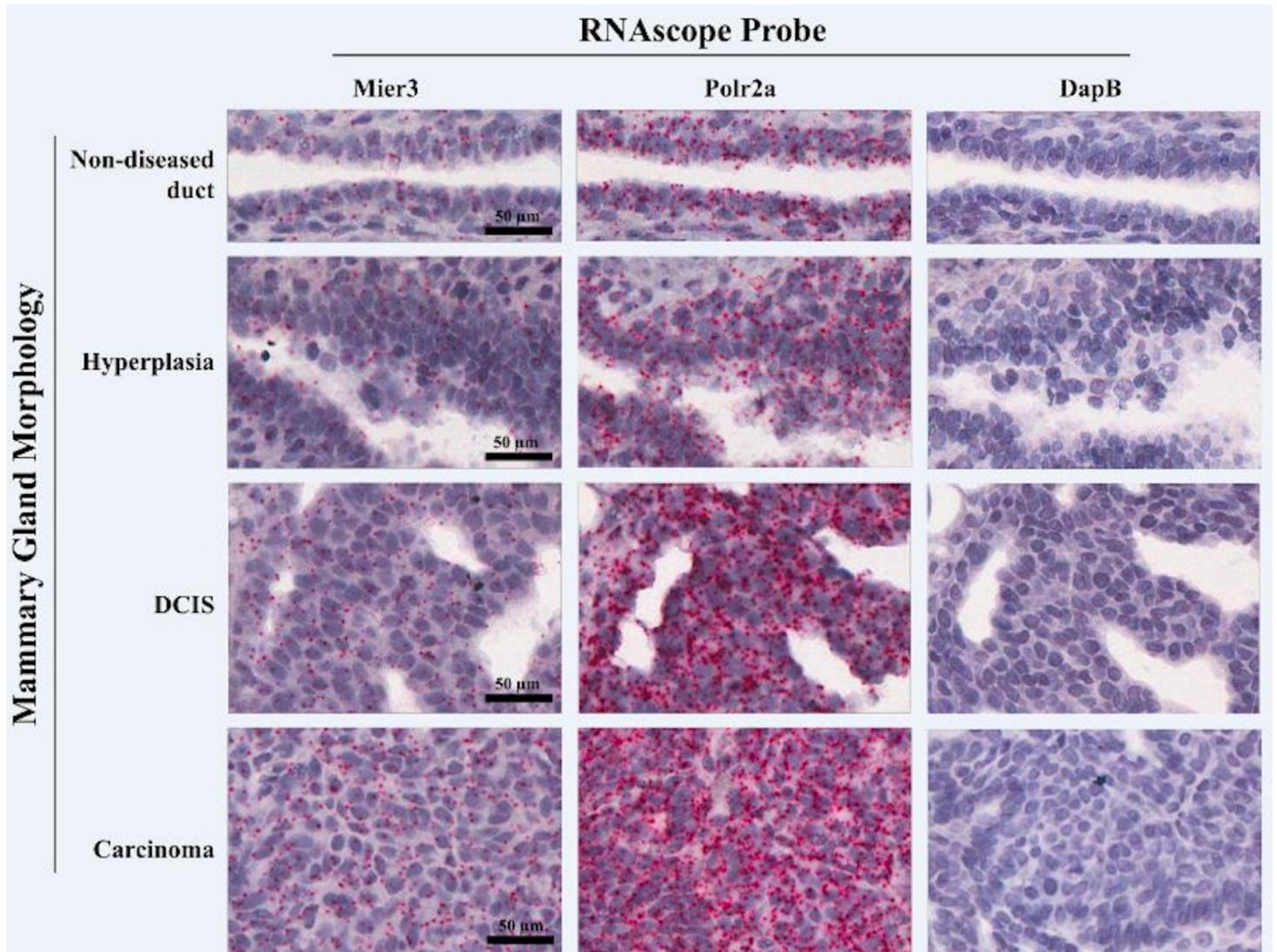
Mammary Gland Tissue Extraction. Left panel: to reveal and expose mammary gland tissue, an incision is made in the skin at the lower abdomen (black circle) and extended along the midline. Four additional incisions are made extending from the midline down each limb. Skin is separated from the body cavity from these incisions. Right panel: Rats have 6 mammary gland pairs along each ventro-lateral boundary of the body, for a total of 12 glands. Conventional labeling begins at cervical glands labeled A in the diagram. This pair of glands is situated next to the salivary glands at the anterior of the animal. Moving toward the posterior are thoracic pectoral glands B and C, abdominal gland D, and inguinal glands E and F.



**Figure 2**

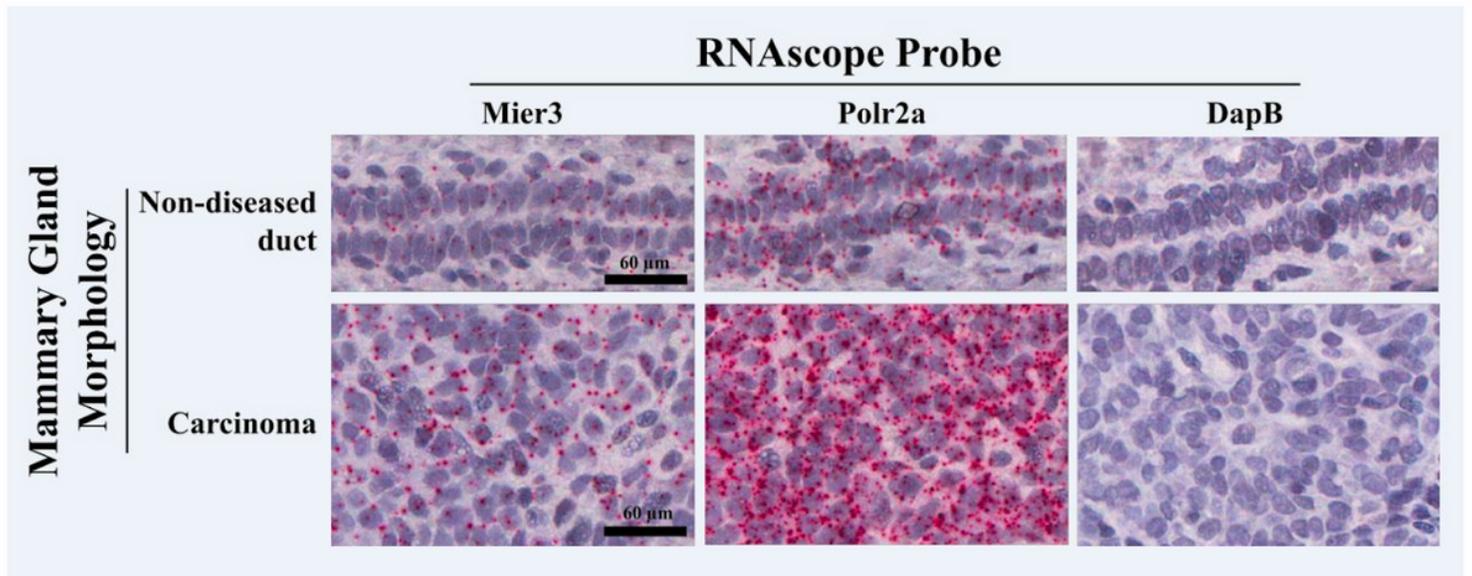
Premalignant and malignant lesions are visible in mammary gland tissue whole mounts following aluminum carmine staining. Shown are representative mammary glands from virgin female Wistar Furth (WF/Hsd) rats taken 8 weeks after administering a chemical carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA). Hyperplasia, induced by DMBA, appears as dark thickened branching (demarcated by black

boxes). Carcinogen-induced DCIS appears as a dark mass within mammary fat pads (marked by black arrows).



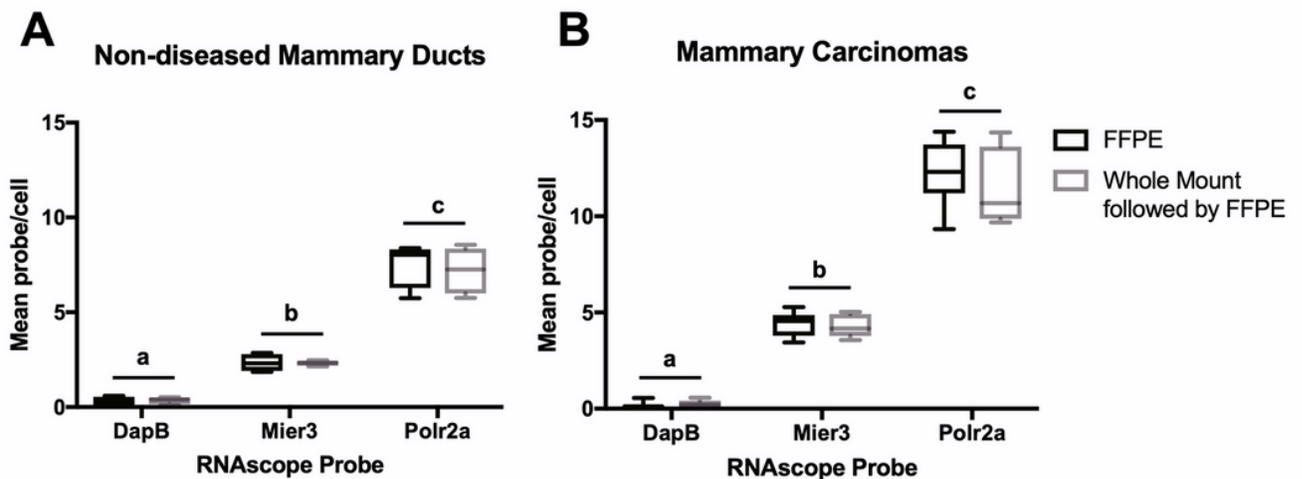
**Figure 3**

Representative images of RNAscope using Mier3, Polr2a, and DapB probes on tissue sections obtained from rat mammary gland tissue whole mount preparations. Rat mammary gland ductal hyperplasia, ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) were first identified in formalin fixed aluminum carmine stained whole-tissue mounts followed by excision and processing. The RNAscope assay performed on these morphologies used Mier3 family member 3 (Mier3) as a moderately expressed gene (left column), RNA Polymerase II Subunit A (Polr2a) as a highly expressed gene (middle column) and bacterial 4-hydroxy-tetrahydrodipicolinate reductase (DapB) as a negative control gene (right column). Histologic images show red puncta counterstained with hematoxylin. Each red puncta represents a single mRNA transcript [5]. Images were taken at 40x magnification on an Aperio ImageScope CS2.



**Figure 4**

Representative images of RNAscope assays using Mier3, Polr2a, and DapB probes on tissue sections of fresh FFPE rat mammary gland and carcinoma tissue. Tissues were formalin fixed and processed immediately following resection (manufacturer recommended). Samples are from non-diseased mammary glands or DMBA-induced mammary carcinomas. RNAscope assay probes for Mier3 family member 3 (Mier3), as a moderately expressed gene (left column), RNA Polymerase II Subunit A (Polr2a), as a highly expressed gene (middle column), and bacterial 4-hydroxy-tetrahydrodipicolinate reductase (DapB), as a negative control gene (right column) were used. Histologic images show red puncta counterstained with hematoxylin. Each red puncta represents a single mRNA transcript [5]. Images were taken at 40x magnification on an Aperio ImageScope CS



**Figure 5**

Quantitative comparison of RNAscope assays using Mier3, Polr2a, and DapB probes on FFPE processed and whole mounted tissues. RNAscope assays were performed on pairs of mammary glands (n=6) (panel A) and DMBA-induced mammary carcinomas (n=6) (panel B) taken from the same rats. Tissues were either immediately processed by FFPE (manufacturer recommended) or whole mounted on microscope slides and stained with aluminum carmine prior to paraffin embedding. Tissue was probed for Mier3 family member 3 (Mier3), a moderately expressed gene, RNA Polymerase II Subunit A (Polr2a), a highly expressed gene, and bacterial 4-hydroxy-tetrahydrodipicolinate reductase (DapB), a negative control gene that should not be present in mammalian tissues. RNAscope probes per cell in non-diseased mammary ducts and mammary carcinomas were quantified using HALO-ISH Quantification software from Indica Labs. A two-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 7.00 Software. In each tissue type, there was no significant difference between processing methods (Panel A-  $p=0.5057$ , Panel B-  $p=0.6086$ ). As expected, there was a significant difference between probes in either non-diseased mammary tissue or mammary carcinomas (2-way ANOVA  $p<0.0001$ ). RNAscope probes with different lowercase letters were significantly different for Tukey's post hoc test ( $p<0.05$ ).