Vital ex vivo tissue labeling and pathology-guided micropunching to characterize cellular heterogeneity in the tissue microenvironment

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Cellular heterogeneity within the tissue microenvironment may underlie chemotherapeutic resistance and response, enabling tumor evolution; however, this heterogeneity it is difficult to characterize. Here, we present a new approach—pathology-guided micropunching (PGM)—that enables identification and characterization of heterogeneous foci identified in viable human and animal model tissue slices. This technique consists of live-cell tissue labeling using fluorescent antibodies/small molecules to identify heterogeneous foci (e.g., immune infiltrates or cells with high levels of reactive oxygen species) in viable tissues, coupled with a micropunch step to isolate cells from these heterogeneous foci for downstream molecular or vital functional analysis. Micropunches obtained from epithelial or stromal fibroblast foci in human prostate tissue show 6- to 12-fold enrichment in transcripts specific for EpCam/cytokeratin 8 and vimentin/ α -smooth muscle actin/integrin 1- α , respectively. Transcriptional enrichment efficiency agrees with epithelial and stromal laser capture microdissection samples isolated from human prostate. Micropunched foci show a loss of cellular viability in the periphery, but centrally localized cells retained viability before and after dissociation and grew out in culture.

Cellular heterogeneity is hypothesized to affect chemotherapeutic resistance and response, enabling tumor evolution, but it is difficult to quantify and characterize (1,2). One source of cellular heterogeneity in a tissue is the cell's response and adaptation to its local microenvironment, which creates clusters of cells exhibiting similar molecular and functional characteristics proximally, but differing from more distal neighboring cells. This microenvironmental heterogeneity can occur in a predictable way when driven by tissue architecture, for instance in the case of periportal and perivascular hepatocytes that harbor extensive differences in drug metabolic activity in response to differ-

ences in oxygen tension. Alternatively, microenvironmental heterogeneity can apparently present at random, driven by unseen molecular changes, such as occurs with treatment-resistant foci within a tumor (3,4). Microenvironmental heterogeneity underlies critical biological functions that are key to understanding human health and disease in many different contexts. The functional nature of this biology and the cellular scale at which this heterogeneity exists (groups of tens to thousands of cells) requires non-traditional targeted approaches to isolate and study these differences.

Historically, histopathology has utilized laser capture microdissection to isolate

specific biomolecules from microscale regions of interest in thinly sliced (<10 μ m thick) sections (5,6). Despite recent improvements, this technique is limited by the need for expensive equipment, low-throughput/long processing times, and experienced operators. Furthermore, laser capture microdissection requires fixation or freezing of tissue, resulting in a non-viable specimen and eliminating the ability to do live-cell functional analyses (7). In addition, multiple areas must often be pooled to obtain sufficient material (RNA, DNA, etc.), which prohibits the characterization of heterogeneity between individual foci (8,9). Alternatively, a tissue punching technique developed over 50

METHOD SUMMARY

Live-tissue slicing and fluorescent labeling is coupled with a micropunch technique to isolate microscale heterogeneous foci for nucleic acid, protein, or functional analyses to explore the underlying biology.

years ago can be used to isolate architecturally distinct foci from frozen and fresh brain slices (10,11). Variations of this technique have been used to isolate foci from sections labeled by RNA in situ hybridization, but this approach doesn't integrate molecular or functional staining to identify heterogeneous foci in viable tissue (12). These limitations present a significant hurdle in identifying and exploring the biological basis for this heterogeneity.

Here, we present a simplified approach, called pathology-guided micropunching (PGM), that uses standard laboratory equipment to identify and isolate subpopulations of viable cells from microscale foci for downstream molecular analysis. Our method utilizes laboratory equipment, including an inverted microscope, glass bottom Petri dish, nylon mesh, and blunt needle/micropunch. Our data demonstrate that we are able to molecularly and functionally interrogate viable whole-mount tissue slices, micropunch areas of interest, and perform gene expression analysis of human tissue specimens. Enrichment of cellular subpopulations was validated by comparing enrichment of epithelial versus stromal transcripts in human prostate tissue punches to standard laser capture microdissection transcriptional profiles from human prostate. This approach can be tailored to preserve molecular integrity or cellular viability of dissociated or intact micropunches.

Materials and methods

Vibratome live sectioning

A detailed step-by-step protocol for PGM can be found in the Supplementary Material. Prostate tissue samples (2–7 mm diameter) were acquired in accordance with the UW-Madison Institutional Review Board guidelines and sectioned on a VT1000S Vibratome (Leica, Wetzlar, Germany) or a VF-300 Compressotome (Precision Instruments, Greenville, NC). Biopsies were superglued to the cutting stage and mounted in 2.5% IBI Scientific Low Melt Agarose (IBI Scientific Peostia, IA) diluted in 1× PBS. The stage was then immersed in Cellgro 1× DMEM (Corning Inc., Corning, NY) sterile media with 1% pen-strep. Following stage preparation, vibratome frequency was set to 80 Hz and speed to 0.5 mm/sec, and 30 to 200 μ m serial sections were taken of the prostate biopsies. Each section was placed inside

Figure 1. Overview of pathology guided micropunching.

a screwtop vial with 75 μ l of DMEM sterile media and placed on wet ice.

Live-cell staining and imaging

Before imaging, desired slices were stained in screwtop vials with fluorescent dyes. Staining protocols for each of the specific dyes (ThermoFisher Scientific, Waltham, MA) included Rapid stain–Hoechst (1.6 mM), *Erythrina cristagalli* lectin–fluorescein (18.5 nM) (Vector Biotech, Burlingame, CA), Phalloidin–Texas Red (66 nM) for 15 min at room temperature. Functional stain included Hoechst (1.6 mM), Cell Rox (6.25 mM), CMX:Ros (1 mM), MitoTracker Deep Red (1 mM) for 30 min at 37°C. Antibody stain included: Hoechst (1.6 mM), CD45–FITC (1:50) (Clone H130; Biolegend San Diego, CA), EpCAM-PE (1:100) (Clone VU-1D9, Abcam Cambridge, MA), and CD49a–Alexafluor 647 (1:100) (Clone TS2/7; Biolegend) for 60 min at 4°C. Following incubation, slices were rinsed twice with 500 μ l sterile media and were laid flat on 50 mm glass bottom dishes (MatTek Corp., Ashland, MA). Nylon Mesh (Component Supply Co., Sparta, TN), with a pore size matched to the sampling needle (i.e., 500 μ m mesh fits the outer diameter of a 25 gauge sampling needle), along with a weighted aluminum ring, was placed over the tissue slices in order to hold them flat for imaging. Before images were taken, medium on the tissue slices was removed, and 1.5 mL RNA Later (Sigma-Aldrich, St. Louis, MO) was pipetted over the samples to preserve RNA integrity. Images were then taken using a 10× objective on an inverted microscope

(BD Pathway Confocal; BD Biosciences, Franklin Lakes, NJ / Nikon Eclipse Ti; Nikon Instruments, Tokyo, Japan). These images were saved for comparison to corresponding 5- μ m H&E slides produced from the tissue samples.

Tissue micropunching

Micropunching was done by hand with the same staging and setup as described above for the imaging of the tissue slices. The nylon mesh and aluminum ring stabilized and held the tissue samples flat against the imaging dish during punching. A piece of 2-mm glass was also placed under the MatTek Corp. imaging plate to provide support, preventing the needle from breaking or delaminating the glass bottom dish. A 2× objective was used on an inverted fluorescent microscope (BD Pathway or NikonA1) in live mode to guide our micropunching needle to desired tissue areas of interest. To aid in ejection of the tissue micropunch, a 1-mL syringe was used to aspirate ~10 μ l 1× PBS into the tip of a 25-gauge blunt-end needle. The 25-gauge needle was used to core out an area of interest from the tissue slice by pressing the needle down and turning it back and forth while the tissue was stabilized by the ring and nylon mesh. The micropunch was ejected using the 1-mL syringe into 100 μ l collagenase/DNase digestion buffer (cells) or TRIzol LS Lysis Reagent (ThermoFisher Scientific, Waltham, MA) inside a 0.2-mL PCR tube. The micropunching process was repeated until all areas of interest had been cored from each of the tissue samples. Micro-

Figure 2. Viable tissue slice staining enables pathology-guided micropunching (PGM). Pseudocolored fluorescence micrographs of vibratome-sectioned slices stained to identify heterogeneously distinct foci for micropunching. (A) Upper panel: Rapid vital staining includes DNA and F-actin intercalating dyes (Hoechst–blue; phalloidin–red, respectively) and lectins (shown: *Erythrina crista-galli* lectin–fluorescein–green). Middle panel: Antibody staining includes fluorescently tagged antibodies for CD45–FITC (immune cell marker, arrow marks duct with immune infiltrate)–cyan; EpCAM–PE (epithelial cell marker)–red, and CD49a–Alexa-647 (fibroblast marker)–green, counterstained with Hoechst–blue. Lower panel: Functional metabolic staining includes CMXRos (mitochondrial membrane potential)–red, far red MitoTracker (total mitochondria)–magenta, and cellROX [reactive oxygen species (ROS); arrow marks duct with high ROS]–green, counterstained with Hoechst–blue. (B) Upper panel: Slice stained as in lower panel of (A), before and after RNAlater was added; micropunched slice is shown before and after FFPE, sectioning, and hematoxylin and eosin (H&E) staining. (C) RNA isolated from micropunches in (B) show enrichment of *GAPDH* and *P0* gene expression compared with the control. Data are representative of three independent experiments.

punched slices were fixed overnight in 10% formalin and transferred to 70% ethanol. Slices were mounted on a glass slide under 2% agar on a 60°C hotplate, and forceps were used to push tissue slices flat against the glass while the slide was removed from the heat in order to allow the agar to gel. Tissue slices underwent a shortened 3-h processing and paraffin embedding.

RNA extraction of tissue micropunches

RNA was extracted from micropunches using TRIzol LS Reagent (Qiagen, Hilden, Germany) followed by a Qiagen MagAttract RNA Universal Tissue M48 Kit customized protocol using the upper aqueous phase. First, 5 μ l MagAttract Suspension E silica beads were added and mixed into each sample. Suspension E beads were separated from the mixture using a magnetic tube rack, and 100 μ l of Wash Buffer MW was added. This process was repeated with another 100 μ l of Wash Buffer MW. Suspension E beads were again separated from the mixture, and the wash buffer was aspirated. Wash Buffer RPE (100 μ l) was added, mixed, and aspirated twice.

Isolated RNA was eluted from Suspension E beads in 25 μ l RNase-free water.

Transcriptional characterization, data mining and statistical analysis

Reverse transcription and preamplification was performed using the High-Capacity cDNA Reverse Transcription Kit and the TaqMan PreAmp Master Mix Kit (both purchased from ThermoFisher Scientific, Waltham, MA), respectively, and the Roche Life Science Light Cycler 480 (Roche, Basel, Switzerland) according to the manufacturer's protocol. The cycle threshold value, C_t , was used to evaluate relative gene expression of epithelial marker proteins EpCAM [Hs00901885_m1] and Cytokeratin 8 [Hs01595539_g1]; immune marker protein CD45 [Hs04189704_m1]; and stromal fibroblast marker proteins integrin 1 α (CD49a) [Hs00235006_m1], α -smooth muscle actin [Hs00426835_g1], and vimentin [Hs00958111_m1]; House-keeping genes *GAPDH* [hs99999905_m1] and *RPLP0* (hs00420895_gH) were used for comparison. The $\Delta\Delta C_t$ method was used to normalize expression across samples using *GAPDH*. A Student's

two-tailed *t*-test was used to test for differences in expression between cellular compartments. Expression was compared with an unpunched blank control taken from the slice suspension media.

Laser capture microdissection microarray data investigating gene expression differences between laser captured prostate epithelial and interstitial stromal cells was mined from NCBI GEO database, accession number GSE20758 (13,14). Probes used were epithelial—EpCAM [201839_s_at] and cytokeratin 8 [209008_x_at]; immune—CD45 [212587_s_at]; and stromal fibroblast markers—integrin 1 α (CD49a) [214660_at], α -smooth muscle actin [200974_at], and vimentin [201426_s_at]; house-keeping—*GAPDH* [212581_x_at] (used for comparison). The GEO Data Analysis Tool was used to generate normalized expression levels for each probe. A Student's two-tailed *t*-test was used to test for differences in expression between cellular compartments.

Results and discussion

Heterogeneity across the tissue microenvironment is driven by tissue architecture, local paracrine signaling interactions, and immune responses that are difficult to recapitulate in vitro and inaccessible when using in vivo models. Development of tools to study these interactions ex vivo and in situ within whole-mount tissue slices could improve our understanding of the underlying biology in many different contexts. Here, we combined methods that identify heterogeneous foci in viable tissue slices with a method that isolates microscale cellular foci for downstream molecular and functional analyses. Critical steps in the procedure, including tissue slicing, fluorescent labeling, sample plating, and micropunching, are illustrated in Figure 1 and discussed below; a detailed protocol is provided in the Supplementary Materials.

Tissue slicing: The majority of live-cell approaches to study tissue ex vivo have been developed for neurological research (15). Fresh tissue samples can be crudely cut with a razor or sliced on a vibratome or compresstome to maximize the tissue surface area analyzed (Figure 1A). We found that cutting slices on the compresstome enabled more robust cutting of a broader ranges of tissue types and thicknesses as compared with a vibratome. At an accepted 10% failure rate, we could consistently section 30- to 400- μ m-thick sections on a VT-300

Figure 3. Pathology-guided micropunching (PGM) enriches epithelial and stromal fibroblast-specific RNA expression compared with laser capture microdissection. Epithelial and stromal fibroblast transcripts are enriched in pathology-guided micropunches and archival laser capture microdissection samples of human prostate. (A) Pseudocolored fluorescence micrographs of EpCAM-high and CD49a-high foci in human prostate slices: EpCAM-PE (epithelial cell marker)–red and CD49a-Alexa-647 (fibroblast marker)–cyan, counterstained with Hoechst – blue, before and after micropunching. (B) Micropunches isolated from epithelial-rich regions in (A) show significant enrichment in the epithelial transcripts EpCAM and CK8, whereas stromal punches are enriched in α -SMA, vimentin and CD49a (two-tailed *t*-test, $P < 0.05$). (C) Laser capture microdissection of epithelial and stromal compartments show similar trends in microarray gene expression compared with PGM.

compresstome, but only 150- to 400- μ m-thick sections on a Leica 1000S vibratome.

Fluorescent labeling: Fluorescently tagged antibodies, chemical stains, and vital fluorescent reporters of cellular function can be used to stain viable whole-mount tissue slices (Figure 1B and Figure 2). Early approaches used a tissue's architectural morphology to define and sample tissue foci in frozen and fresh brain slices (10,11), but heterogeneity unrelated to tissue architecture, such as molecular and functional changes (i.e., metabolic, cellular senescence), are important for tumor evolution and therefore require alternative approaches. Whole-mount tissue slice staining or imaging of endogenous fluorescence are emerging

techniques used to identify heterogeneous foci in viable tissue slices, such as those generated by a vibratome (16,17).

We developed staining techniques for tissues from both animal models, as well as clinical patient prostatectomy samples, which are the subject of most of the data presented. In our studies, antibody and chemical stains were used to identify cancerous regions and provide a topographical understanding of the cellular microenvironment (Figure 2). To identify static histological landmarks, such as normal and tumor prostate epithelial cells, we used fluorescent antibodies reported in the literature [typically used for standard formalin-fixed, paraffin-embedded (FFPE) sections] to discern these cell types. The benefits of

staining viable cells include reduced autofluorescence and improved accessibility of antigens due to the lack of protein cross-linking during fixation (18). While we have performed live-cell permeabilization with Triton, targeting extracellular membrane proteins led to far shorter staining incubation times (30 min versus 3 h) and decreased background staining.

Vital functional stains, which are typically used for cultured cells, have been shown to work in ex vivo tissues as well as in vivo (16,19). Vital functional stains for total mitochondria, mitochondrial membrane potential, glucose uptake, and reactive oxygen species (ROS) detection were used to read out the metabolic state across the slice (Figures 2 and 4). In our experience, functional stains were more likely to identify heterogeneous regions within the slices (Figure 2 and Supplementary Figure S1). Tissue autofluorescence from collagen and lipofuscin were also commonly seen in prostate tissues, requiring adjacent no-stain control slices for each stained patient slice. Collagen autofluorescence appears diffuse, with 488/530 nm excitation/emission at 10 $\times$ magnification, but more fiber-like at higher magnification (20). Lipofuscin fluoresces equally in the 488/530 nm and 560/590 nm channels, appearing as punctate granules in the epithelial ducts of prostate tissue. Lipofuscin levels have been shown to be prognostic for patient outcomes in low-grade prostate cancer, highlighting that the utility of endogenous autofluorescence should not be overlooked (21).

Sample plating, imaging and micropunching: Plating the stained slices for imaging and micropunching was the most critical step in the process and is described in detail in the protocol (Figure 1C). The sample was imaged using a 10 $\times$ or 20 $\times$ objective to identify foci for micropunching (Figure 1D). A 2 $\times$ or 4 $\times$ objective was then used on an inverted fluorescent microscope (BD Pathway or NikonTi) in live mode with a 647/683 nm excitation/emission filter to guide our micropunching needle to microscale regions of interest (equivalent to the size of the eye on a Lincoln penny) (Figure 1E). The longer wavelength imaging channels are preferable when sampling, due to reduced phototoxicity and photobleaching. We could micropunch at a rate of approximately 1 micropunch per 1–2 min once the slices were imaged and regions of interest (ROIs) were identified. Images taken before, after, and during micropunching of

both human prostate and mouse liver tissue slices are shown in Figures 2, 3, and 4.

Post-sampling analysis: The PGM protocol can be altered to facilitate cellular viability or stabilize sensitive molecules such as nucleic acids. For the molecular analysis of RNA, DNA, and protein, we sought to limit molecular changes caused by ex vivo sample processing. Placing slices in the nucleic acid preservative RNAlater did not have a detrimental effect on the stains we tested, provided staining was done before, not after, placement in RNAlater (Figure 2B). We also found that the remaining tissue could be fixed and sent for histological processing (Figure 2B), consistent with other reports where RNAlater treatment was shown not to harm tissue morphology (22). The ability to tie novel molecular and functional staining approaches with standard hematoxylin and eosin (H&E) histopathology is critical to generate read-across to standard-of-care clinical approaches. Micropunches isolated from RNAlater-treated slices showed good agreement of RNA-specific expression of the housekeeping genes *GAPDH* and *P0*. Across the 3 patients, micropunches showed on average a 5 C_q increase (32-fold enrichment) over a blank volume taken from the dish (representative data are shown in Figure 2C and Supplementary Figure S1). Zero expression in the blank sample would be ideal, but that is not expected given that the tissues are submerged in the blank solution. In these studies, 34 of 36 samples (94.4%) showed >10-fold enrichment over background and therefore were considered successful. In some cases, the ejected micropunch was not seen in the collection tube. This suggests that the two low-expressing samples could have been lost in the micropunching needle or tube.

To quantify the cellular enrichment efficiency of PGM, human prostate slices were stained and micropunched in areas with high epithelial (EpCAM) or stromal fibroblast (CD49a) staining (Figure 3A). Micropunches were isolated from the epithelial and stromal fibroblast regions, then lysed for RNA isolation and expression quantification by qRT-PCR. After normalization to the housekeeping genes *GAPDH* and *P0*, transcripts specific to epithelial cells, *EpCAM* and cytokeratin 8, showed an average 6- and 8-fold enrichment, respectively, over the mean expression in stromal punches ($P < 0.05$) (Figure 3B). The stromal fibroblast markers integrin 1 α (CD49a), α -smooth muscle actin, and vimentin showed on

average 12-, 10- and 12-fold enrichment, respectively, in stromal punches compared with the mean expression in epithelial punches ($P < 0.05$) (Figure 3B).

To further understand the PGM cellular enrichment results, we mined archival laser capture microdissection expression data from epithelial and stromal regions in human prostate (14). After normalization to *GAPDH*, *EpCAM* and cytokeratin 8 showed 21-fold and no enrichment, respectively, in laser-captured epithelial foci over the mean expression in stromal foci ($P < 0.05$) (Figure 3C). The stromal fibroblast markers integrin 1 α (CD49a), α -smooth muscle actin, and vimentin showed on average 3-, 6- and (non-significant) 3-fold enrichment, respectively, in the stromal laser-captured foci compared with the mean expression in epithelial foci ($P < 0.05$). Differences in the methods used to generate these data, including RNA isolation and expression quantification, as well as the use of unmatched patient samples, limit comparisons to simple fold-change between cellular compartments. Yet, these results help put the effectiveness of PGM in context with laser capture microdissection using clinically relevant human prostatectomy samples.

The greatest advantage of PGM is the ability to maintain cellular viability for subsequent analyses ex vivo. To confirm the utility of this approach for maintaining cellular viability, we evaluated cell viability via acridine orange/propidium iodide (AO/PI) staining. Both stains are DNA intercalators, but only AO can enter viable cells, generating green fluorescent nuclei. In dead cells, PI also enters nuclei where fluorescence resonance energy transfer (FRET) of green emitted light from AO excites PI, causing a red-light emission instead of green. Using the AO/PI method, enzymatic digestion of micropunches (300 μ m) isolated from unstained human prostate slices yielded ~85% viable cells (Figure 4A). Tissue slices stained in situ with AO/PI show high viability (Figure 4B). A micropunch isolated from the slice in Figure 4B shows viable cells only in the center of the micropunch, indicating damage to peripheral cells during PGM, whereas all viability is lost in the matched dead-cell control (Figure 4C). Culturing tissue micropunches yielded viable cells emanating from the micropunch after 4 weeks in culture (Figure 4D).

To evaluate the viability/utility of PGM beyond human prostate tissue, we performed enzyme-based (collagenase/DNase) digestion of micropunches

Figure 4. Micropunching viable cells from fresh tissue slices. Viability staining of intact and dissociated micropunches. (A) Enzymatic digestion of micropunches yields both viable and dead cells as measured by the FRET-based viability markers acridine orange (live nuclei—green) and propidium iodide (dead nuclei—red) ($n = 5$). (B) Prostate tissue slices stained with AO/PI show uniform viability before micropunching. (C) Left: Micropunch from (B) shows cell death in the periphery caused by punching. Right—dead cell control, total loss of viability after 24 h uncultured. (D) Micropunch cultured for 28 days shows viable primary cells emanating from the punch. Live cells—green, dead cells—red. (E) Upper panel: A 150- μ m vibratome slice of mouse liver shown before (left), in-process, and post micropunching (right) on an inverted microscope with a 4 $\times$ objective. Lower panel: Increasing vibratome slice thickness showed a linear trend in live cellular yield measured by trypan dye exclusion of cells from digested micropunches ($R^2 = 0.6542$).

isolated from viable mouse liver slices and counted live dissociated cells via trypan blue exclusion. As expected, we found that increasing slice thickness showed an increasing trend in the average number of viable cells per micropunch (Figure 4E). The increased variability of the cell yield in the 75- μ m slices may reflect the difficulties in consistently cutting 75- μ m slices on the vibratome as discussed above. The ability to identify and isolate viable sub-populations from whole tissue provides the ability to perform extensive further characterization of heterogeneous foci.

Comprehensive molecular and functional analyses to characterize microenvironmental tissue heterogeneity requires a reconceptualization of standard approaches to tissue processing. Laser capture microdissection is the most widely used technique to isolate microscale foci within a tissue, but this approach is not compatible with vital end points. We have overcome this challenge through the use of a low-cost, simplified PGM methodology, which combines whole-mount sectioning and staining with a simple and robust micropunching approach that is guided by a fluorescence microscope. We have shown that we can identify molecular and functional heterogeneity within live tissue slices, isolate these microscale foci, and perform gene expression analysis or viable cell isolation to explore the underlying biology of heterogeneity. The remaining tissue is available for complementary histology or other molecular analyses. Application of this technique can advance our understanding of cellular heterogeneity across the tissue microenvironment that leads to tumor evolution, drug resistance/response, and other states of health and disease.

Author contributions

B.P.J. designed the experiments. B.P.J., R.A.V., and P.G.G. performed the experiments. D.F.J., J.M.L., and W.H. provided clinical expertise on sample acquisition and scoring. B.P.J., R.A.V., and D.J.B. wrote the paper.

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Competing interests

David J. Beebe holds equity in Bellbrook Labs, LLC, Tasso, Inc., Stacks to the Future, LLC, Salus Discovery, LLC, Lynx Biosciences, Inc., and Onexio Biosystems LLC. Brian Johnson holds equity in Onexio Biosystems LLC. Joshua Lang holds equity in Salus Discovery, LLC.

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