Oct 10, 2022

Light-Seq

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ABSTRACT

We present Light-Seq, an approach for multiplexed spatial indexing of intact biological samples using light-directed DNA barcoding infixed cells and tissues followed by ex situ sequencing. Light-Seq combines spatially targeted, rapid photocrosslinking of DNA barcodes onto complementary DNAs in situ with a one-step DNA stitching reaction to create pooled, spatially indexed sequencing libraries. This light-directed barcoding enables in situ selection of multiple cell populations in intact fixed tissue samples for full-transcriptome sequencing based on location, morphology or protein stains, without cellular dissociation. Applying Light-Seq to mouse retinal sections, we recovered thousands of differentially enriched transcripts from three cellular layers and discovered biomarkers fora very rare neuronal subtype, dopaminergic amacrine cells, from only 4–8 individual cells per section. Light-Seq provides an accessible workflow to combine in situ imaging and protein staining with next generation sequencing of the same cells, leaving the sample intact for further analysis post-sequencing.

DOI

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PROTOCOL CITATION

Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin 2022. Light-Seq. **protocols.io** https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

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HHMI

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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PROTOCOL INTEGER ID

52794

MATERIALS TEXT

PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Invitrogen - Thermo Fisher Catalog #AM9625**

☒ DNAse/RNAse free distilled water **Invitrogen Catalog #10977023**

☒ Maxima H Minus Reverse Transcriptase (200 U/µL) **Thermo Fisher Catalog #EP0753**

☒ RNaseOUT™ Recombinant Ribonuclease Inhibitor **Invitrogen - Thermo Fisher Catalog #10777019**

☒ Triton X-100 **Sigma Aldrich Catalog #T8787-50ML**

☒ Terminal Transferase - 2,500 units **New England Biolabs Catalog #M0315L**

☒ 2'3'-Dideoxyadenosine 5'-Triphosphate 100 mM solution **Sigma Aldrich Catalog #GE27-2051-01**

☒ Deoxynucleotide Solution Set - 25 umol of each **New England Biolabs Catalog #N0446S** (only need dATP)

☒ Deoxynucleotide Solution Mix - 8 umol of each **New England Biolabs Catalog #N0447S**

☒ Ethyl alcohol, Pure 200 proof, for molecular biology **Sigma Aldrich Catalog #E7023**

☒ Qubit™ 1X dsDNA High Sensitivity (HS) and Broad Range (BR) Assay Kits **Invitrogen - Thermo Fisher Catalog #Q33230**

☒ Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #AM9760G**

☒ Formamide (Deionized) **Thermo Fisher Catalog #AM9342**

☒ Bst DNA Polymerase Lg Frag - 8,000 units **New England Biolabs Catalog #M0275L**

☒ RNase H - 1,250 units **New England Biolabs Catalog #M0297L**

☒ KAPA HiFi DNA Polymerase **Kapa Biosystems**

☒ SYBR™ Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO **Thermo Fisher Catalog #S7563**

☒ Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

☒ 10 x 2 ml IDTE pH 7.5 (1X TE Solution) **IDT Catalog #11-01-02-02**

☒ Tween 20 **Sigma Aldrich Catalog #P9416-50ML**

☒ Pierce™ 16% Formaldehyde (w/v), Methanol-free **Thermo Fisher Catalog #28908**

☒ SecureSeal™-SA8S-1.0 8-7mm X 7mm X 1.0mm Depth **Grace Bio-Labs Catalog #621102** OR

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

☒ µ-Slide 8 Well Poly-L-Lysine Coated #1.5 Polymer Coverslip **Ibidi**

☒ Poly-D-Lysine Hydrobromide **Millipore Sigma Catalog #P6407-5MG**

☒ Pierce™ 20X Borate Buffer **Thermo Fisher Catalog #28341**

☒ Tissue-Tek OCT **Fisher Scientific Catalog #14-373-65**

☒ ThermoPol Reaction Buffer Pack - 6.0 ml **New England Biolabs Catalog #B9004S**

☒ Sucrose molecular biology grade **Sigma Aldrich Catalog #S0389**

☒ Nextera DNA library preparation kit 24 samples **Contributed by users Catalog #FC-121-1030**

☒ Sheared Salmon Sperm DNA 10mg/mL **Thermo Fisher Scientific Catalog #AM9680**

☒ Dextran Sulfate 50% Solution **Sigma Aldrich Catalog #S4030**

Equipment

Qubit
Flurometer
Invitrogen **Q33228**

(or equivalent for measuring dsDNA concentration)

Eppendorf Mastercycler® nexus flat
Eppendorf **71003-568**

Magnetic Separator
10X Genomics **120250**

(or equivalent)

Microscope capable of delivering UV light to regions of interest.

Oligos

Note: Not all S5XX.GATE and Next.N7XX primers are needed. Consult protocol for clarification.

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A	B	C	D	E
Name	**Description**	**Sequence**	**Supplier**	**Purificati**
RT.5N.3G	RT Primer	TTTACACGATTGAGTTATNNNNNGGG	IDT	HPLC
GATE.D12.B1	Barcode sequence 1 - Cy5 labeled barcode strand.	GGAGTTGGAGTGAGTGGATGAGTGATGDDDDDDDDDDDDDTATGGATGAGTTATATAACTCA[cnvK]TCGTGTAAAT[Cy5-3]	GeneLink	PAGE
GATE.D12.B2	Barcode sequence 2 - Cy3 labeled barcode strand.	GGAGTTGGAGTGAGTGGATGAGTGATGDDDDDDDDDDDDDGTTAGGTGAGTTATATAACTCA[cnvK]TCGTGTAAAT[Cy3-3]	GeneLink	PAGE
GATE.D12.B3	Barcode sequence 3 -Fluorescein (FITC) labeled barcode strand.	GGAGTTGGAGTGAGTGGATGAGTGATGDDDDDDDDDDDDDAGGGTATGAGTTATATAACTCA[cnvK]TCGTGTAAAT[Fl-3]	GeneLink	PAGE
GATC.20T	Primer for Cross-Junction Synthesis	GAGAATGTGAGTGAAGATGTATGGTGATTTTTTTTTTTTTTTTTTT	IDT	HPLC
GATE	PCR Primer 1	GGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
GATC	PCR Primer 2	GAGAATGTGAGTGAAGATGTATGGTGA	IDT	HPLC
P5.GATE	Custom Read 1 Primer -required for sequencing ofamplicons.	CGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
GATE*.P5*	Custom i5 index primer -required for some Illuminasequencers (see caption).	CATCACTCATCCACTCACTCCAACTCCGGCG	IDT	HPLC
S502.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACCTCTCTATCGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
S503.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACTATCCTCTCGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
S505.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGCGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
S506.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACACTGCATACGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
S507.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACAAGGAGTACGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
S508.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTCGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
Next.N701	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG	IDT	HPLC
Next.N702	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG	IDT	HPLC
Next.N703	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG	IDT	HPLC
Next.N704	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG	IDT	HPLC
Next.N705	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG	IDT	HPLC
Next.N706	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG	IDT	HPLC

Protocol Overview

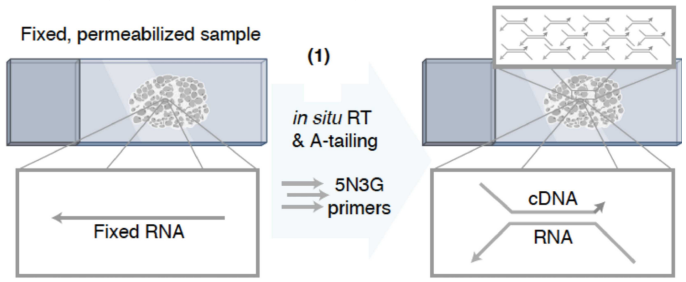
1 This protocol is for performing Light-Seq in tissue samples, as published in Nature Methods. The typical experiment can be performed by a single scientist in one work week, with natural pause points.

Day 0:

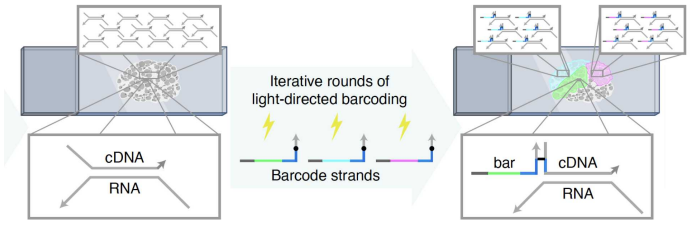
Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

Tissue Dissection & Fixation (~2 hours)

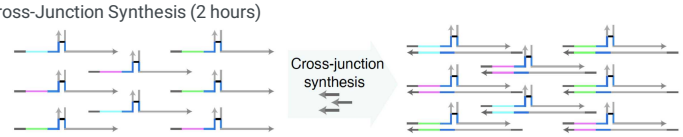
Day 1:
Tissue Sectioning (1 hour)
In Situ Reverse Transcription (2.5 hours)
A-tailing (1 hour)
Antibody Stain (optional, 1-3 hours or overnight)



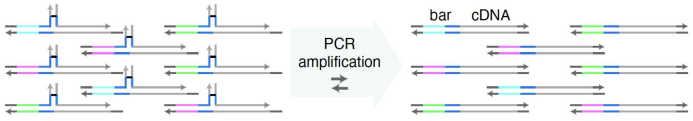
Day 2:
Light-Directed Barcoding (~4 hours for 3 ROIs and 3-6 replicates)



Day 3:
cDNA Displacement (1 hour)
Cross-Junction Synthesis (2 hours)



Day 4:
qPCR and PCR Amplification (5 hours)



Day 5:
Library Preparation for Next-Generation Sequencing (3 hours)

Before Starting

2 This protocol is for selective sequencing of cells within fixed tissue sections that have been sectioned on to a coverslip or microscope slide.

Clean workspace (bench, pipettes, etc.) with ethanol before starting. When the protocol calls for water, always use UltraPure water. All reagents should be molecular-grade and RNAse free. All reactions with enzymes are prepared on ice.

Key Equipment:
Microscope
Light-Seq requires an optical system that can focus UV light onto specific regions of interest. In the publication, we employ a wide field microscope with a 365 nm LED and a digital micromirror device (DMD) to accomplish this, but other systems are also amenable. We have also tested a point-scanning confocal microscope with a 405 nm laser. We recommend consulting your microscopy core or representative to discuss the best solution at your institution.

Flat-top Thermocycler
The first few steps of the protocol require thermal cycling and incubations of tissue slides. For this, we recommend the Eppendorf® Mastercycler® nexus Flat Thermal Cyclers (VWR Cat No. 71003-568).

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

Tissue Dissection and Fixation

3 **Determining tissue-specific fixation/preservation protocol**

The protocol outlined here is for cryo-sectioned mouse retinas. Different tissues require different optimizations for fixation and sample preparation. We highly recommend using standard tissue-specific protocols that are compatible with RNA detection (FISH, for example) as a starting point for Light-Seq.

Times for fixation, washing, and incubation will vary for different sample types. In general, we find that over-fixation can greatly impair Light-Seq barcoding efficiency and in some cases, antigen retrieval protocols are required (e.g. for FFPE). It is recommended to minimally but sufficiently fix your tissue and to freeze immediately to preserve RNA quality. **As a starting point, we recommend starting with standard tissue-specific protocols that are compatible with in situ RNA FISH.**

For users who have not performed FISH in their samples before, we recommend testing the RNA quality in your tissue before proceeding with Light-Seq. This could be done using any number of RNA-detection methods, including HCR, RNA-scope, etc. We expect that tissue preparation parameters that work well for these in situ RNA detection methods should also work well for Light-Seq.

4 **Tissue Fixation and Embedding Reagents**

A	B	C
Reagents	**Supplier**	**Cat. No.**
Triton-X-100	Sigma Aldrich	T8787- 50ML
Pierce™ 16% Formaldehyde (w/v), Methanol-free	Thermo Scientific	28908
PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free	Invitrogen	AM9625
Tissue-Tek® O.C.T. Compound	Sakura	4583
Sucrose, Molecular Biology Grade	Thermo Scientific	J65148.A1

Retina dissection, fixation and embedding

1. Prepare solutions:

4% PFA, 0.25% TritonX-100 in 1X PBS (40 ml):

10 mL 16% PFA ampule
4 mL 10X PBS (molecular-grade)
26 mL nuclease-free water
100 µl Triton-X-100

***Note**: Buffer should be prepared in fume hood as formaldehyde is toxic. Please prepare this fresh and do not freeze.*

30% sucrose in 1X PBS:

15 mg sucrose
in final volume of 50 mL 1X PBS

***Note**: Filter-sterilize using 0.4 µm filter and store at 4C.*

7% sucrose in 1X PBS:

3.5 mg sucrose
in final volume of 50 mL 1X PBS

***Note**: Filter-sterilize using 0.4 µm filter and store at 4C.*

Cryoprotectant Solution:

50 mL of 1:1 mixture of [30% sucrose in 1X PBS] : [Tissue-Tek O.C.T. Compound]

***Note**: This mixture will take time to mix thoroughly. Vortexing will help, but be sure to spin it down after to remove bubbles before applying to the tissue.*

2. Dissect mouse retinas in **1X PBS** at room temperature.
3. Immediately transfer retinas to **4% PFA, 0.25% TritonX-100 in 1X PBS** for 25 mins at room temperature, rocking.
4. Wash 3 x 5 min in 1X **PBS**.
5. Transfer retinas to **7% sucrose in 1X PBS** for 10 min, rocking.
6. Transfer retinas to **Cryoprotectant Solution** for 30 min rocking, to equilibrate the tissue in cryoprotectant before freezing.
7. Transfer retinas to cryomolds and freeze in **Cryoprotectant Solution.**
8. Store frozen retinas at -80C for up to 6 months (possibly longer, but not tested).

Tissue Sectioning and Reverse Transcription

5 **Recommended to section onto a coverslip**

For Light-Seq, it is critical that light can be focused onto the cells of interest. Thus, there should be minimal disruption of the light-path from the microscope to the sample during barcoding. This is very important for labelling isolated cells within a tightly packed tissue, where barcoding boundaries must be precise. In the publication, we

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sectioned retinas into chambers on glass coverslips coated with poly-D-lysine to promote tissue adhesion (Ibidi, #80826). We recommend sectioning onto coverslips if possible, and either using the Ibidi chambers or a chamber that sticks onto the coverslips (GraceBio Labs SecureSeal™ Hybridization Chambers https://gracebio.com/product/secureseal-hybridization-chambers-621102/). These come in a variety of sizes and allow multiple tissue sections to be treated separately on the same coverslip. For these, it is important to cover the holes during extended incubations to prevent evaporation (e.g. with a coverslip or a sticker).

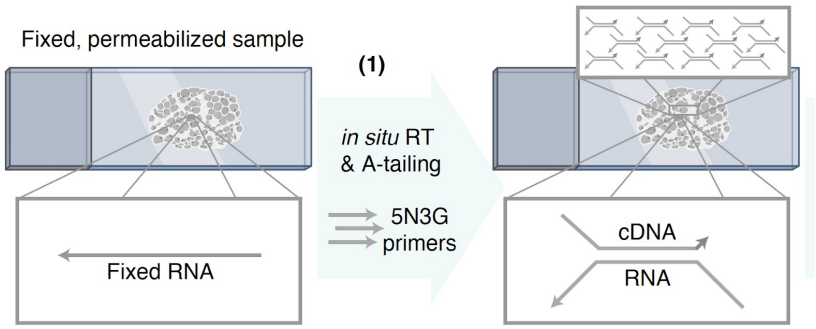
Some tissues do not adhere well to coverslips. If problems occur with tissue adhesion, we recommend coating the coverslip prior to sectioning with a poly-D-lysine solution or some other polymer, baking the sample after sectioning, or sectioning onto SuperFrost Plus slides.

For sections on thick microscope slides

It is possible to perform barcoding on tissue sections that are on SuperFrost Plus Adhesion Slides (Thermo Scientific, #J1800AMNZ). We have found that SuperFrost Plus slides promote better tissue adhesion than coated coverslips. In this case, we recommend using the Grace Bio-Labs SecureSeal Chambers for easy fluid exchanges and to prevent evaporation. However, please note that if barcoding is performed through a thick glass slide then there will be refraction of light through the glass, which can create imprecise barcoding. This is not an issue for barcoding of large regions where the boundaries need not be precise, but for boundary cases where precision of <10 um in the XY-dimension is critical, we do not recommend barcoding through thick slides.

5.1 Cryosectioning and Reverse Transcription

Sectioning for Light-Seq is ideally performed on a coverslip, to promote optimal light delivery for photocrosslinking of DNA barcodes. *In situ* reverse transcription (RT) is performed to create cDNA copies of all RNA within the fixed tissue sections and is done using a thermocycler for slides, rather than tubes. We recommend the Eppendorf® Mastercycler® nexus Flat Thermal Cyclers (VWR Cat No. 71003-568).



Reverse transcription is performed *in situ* using random primers (5N3G) with a barcode docking site on a 5' overhang. This dock site enables selective cDNA barcoding via photocrosslinking in downstream steps.

Reagents for Sectioning

A	B	C
Reagents	**Supplier**	**Cat. No.**
Option 1: µ-Slide 8 Well, Poly-L-Lysine coated	ibidi	80826
Option 2, Component 1: Grace Bio-Labs SecureSeal Hybridization Chambers	Grace Bio-Labs	621102
Option 2, Component 2: LabScientific Rectangular Coverslips, 24 x 50 mm, #1.5, 60 pack	Fisher Scientific	LabScientific 7816
TWEEN® 20	Sigma Aldrich	P9416- 50ML
PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free	Invitrogen	AM9625
20X Borate Buffer	Thermo Fisher	28341
Poly-D-lysine hydrobromide	Millipore Sigma	P6407-5MG
UltraPure Water	Invitrogen	10977023

We recommend either sectioning into an Ibidi chamber (Option 1), or sectioning onto a coverslip with a Grace Bio-Labs chamber on it (Option 2, two components).

Reagents for Reverse Transcription

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

A	B	C
Reagents	**Supplier**	**Cat. No.**
Thermo Scientific™ Maxima H Minus Reverse Transcriptase (200 U/µL) (includes 5X buffer)	Thermo Scientific	FEREP0753
Triton-X-100	Sigma Aldrich	T8787- 50ML
5M NaCl	Invitrogen	AM9760G
TWEEN® 20	Sigma Aldrich	P9416- 50ML
PBS	Invitrogen	AM9625
Formamide	Invitrogen	AM9342
UltraPure Water	Invitrogen	10977023
Deoxynucleotide (dNTP) Solution Mix - 8 µmol at 10mM each	NEB	N0447S
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Invitrogen	10777019

Reverse Transcription DNA Oligos

A	B	C	D	E
Name	**Description**	**Sequence**	**Supplier**	**Purification**
RT.5N.3G	RT Primer	TTTACACGATTGAGTTATNNNNNGGG	IDT	HPLC

Recommended to dilute this primer to a 10 uM stock.

Section and perform in situ reverse transcription

1. Prepare solutions:

1X PBS (40 mL):
4 mL 10X PBS
36 mL UltraPure water

0.1% PBS-Tw (30 mL):
30 mL 1X PBS
30 µL Tween-20

PDL Solution
0.3 mg/mL poly-D-lysine dissolved in 2X Borate Buffer
Note: Store in aliquots at -20C. Do not freeze-thaw.

Stringent Wash (0.1% PBS-Tw + 60% formamide):
3 mL 10X PBS
18 mL 100% formamide
9 mL UltraPure H2O
30 µL Tween-20
Note: *Store at 4C for use later during barcoding.*

High-Salt Wash (1X PBS + 1 M NaCl + 0.1% Tween- 20):
4 mL 10X PBS
8 mL 5 M NaCl
28 mL UltraPure H2O
40 µL Tween-20
Note: *Store at room temperature for use later during barcoding.*

10% Triton X-100 (100 µl):
10 µL Triton X-100
90 µL UltraPure water
Note: Mix very well by vortexing. Recommended to make this in a 2 mL tube for better mixing.

2. Coat coverslip with PDL.
- For Ibidi Chambers: Pipette **PDL Solution** onto the coverslip and let sit for 2 hours or overnight at 4C. Then remove **PDL Solution**, allow slide to dry completely, and wash once with UltraPure water.
- For SuperFrost slides or coverslips: Pipette 500 µL of **PDL Solution** onto the slide and let it sit for 1min. Then remove all liquid, allow slide to dry completely, and wash once with UltraPure water.

3. Trace locations of Grace BioLabs Chambers on the back of the coverslip to guide section placement.
4. Prepare **Reverse Transcription Master Mix** (**RTMM**) on ice, leaving out enzymes (Maxima RT H minus and RNAse Out). Enzymes will be added directly before RT.

Reverse Transcription Master Mix (RTMM)

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A	B	C
Reagent	**Reaction concentration**	**µL reagent per 50µL reaction**
5X RT buffer	1x	10
10 mM dNTPs	300 µM	1.5
UltraPure water		26
10% Triton X-100	0.5%	2.5
10 µM RT.5N.3G primer	1 µM	5
100 mM RnaseOUT	**6 mM**	**3**
Maxima RT H Minus (200 U/uL)	**8 U/µL**	**2**
Total		**50**

Note: Leave out RnasaOUT and Maxima RT H Minus until Step 13.

5. Section tissue on a cryostat, thickness of 5-18 µm.
6. Once all sections are on the coverslip, bring to room temperature.
7. Immediately spin the coverslip in a plate-centrifuge at 600g for 3 min to promote tissue adhesion.
8. Allow sections to dry completely at room temperature (~10min, time depends on section thickness).
9. Wash sections 3 x 1 min with 1X PBS to fully remove cryoprotectant.
10. Allow coverslip to dry completely between sections (use aspirator or Kimwipe).
11. Adhere Grace BioLabs Chambers to coverslip.
12. Wash sections 3 x 1 min with 0.1% PBSTw.
13. Add enzymes (Maxima RT H minus and RNAse Out) to **RTMM** and mix well.
14. Remove 0.1% PBSTw and add 50 ul of **RTMM** to each well.
15. Place slide into Eppendorf® Mastercycler® nexus Flat and run RT program (2h 20 min). Place a second coverslip over the chambers to prevent evaporation.

Reverse Transcription Thermocycler Program
12-cycle ramp program (Lid: 60°C):
Phase 1:
22°C 30 mins
Phase 2 (**12 cycles**):
8°C 30 s
15°C 30 s
25°C 30 s
30°C 1 min
37°C 1 min
42°C 2 min
Phase 3:
42°C 30 min
4°C Forever

16. Wash each well 3 x 5 min in **Stringent Wash**.
17. Wash each well 2 x 2 min in **High Salt Wash.**
18. Wash each well 2 x 2 min in **0.1% PBS-Tw.**
19. Fill each well with 50 ul of **0.1% PBS-Tw.**

Optional Pause Point: Store at 4C overnight before A-tailing.

NOTE: Sectioning for the publication was done immediately before reverse transcription. It is possible, however, to section and immediately freeze the sections at -80C. If using frozen sections, start from step (8) above, and allow sections to dry completely after thawing. To preserve RNA quality, the time between section thawing and RT should be minimal.

A-tailing 45m

6 After *in situ* RT, A-tailing is performed to add a polyA tail to the 3' end of newly transcribed cDNAs, providing a handle on the 3' end for downstream amplification. The A-tailing reaction is isothermal at 37C, and can be performed in a flat-top thermocycler or in a hybridization oven. Once cDNAs are A-tailed, immunofluorescent staining can optionally be performed to identify cells before barcoding.

A-tailing Reagents

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

A	B	C
Reagents	**Supplier**	**Cat. No.**
Deoxynucleotide (dNTP) Solution Set- 25 µmol each at 100 mM (just need dATP)	NEB	N0446S
2',3'-Dideoxyadenosine 5'-Triphosphate, 100 mM solution(ddATP)	Sigma Aldrich	GE27-2051-01
Terminal Transferase Enzyme (TdT) - 2,500 units	NEB	M0315L
ThermoPol® Reaction Buffer Pack	NEB	B9004S
TWEEN® 20	Sigma Aldrich	P9416- 50ML
10X PBS	Invitrogen	AM9625

Perform A-tailing, *in situ*

1. Create aliquots of 10 mM dATP (20 µL aliquots) and 25 mM ddATP (~3 µL aliquots). Store at -20C.
2. Dilute ddATP stock to 250 µM (1:100 dilution from 25 mM stock).
3. Create **A-tailing Master Mix** on ice:

A	B	C
Reagent	**Reaction concentration**	**uL reagent per 50uL reaction**
10X ThermoPol Reaction Buffer	1X	5
10 mM dATP	1 mM	5
250 µM ddATP	25 µM	5
Ultrapure water		32.5
TdT enzyme (20,000U/mL)	1000 U/mL	2.5
Total		**50**

4. Wash wells once with **0.1% PBS-Tw.**
5. Aspirate, add 50 µL **A-tailing Master Mix** and incubate for 45min at 37C. If using Grace Bio-Labs chambers, be sure to cover the holes with a coverslip or stickers to prevent evaporation.
6. Wash 3 x 1min in **0.1% PBS-Tw**.

Optional Pause Point: Store at 4C overnight before barcoding.
Step 6 includes a Step case.
Immunostaining

Light-Directed Barcoding of cDNAs

step case

Immunostaining

If you want to perform immunofluoresence to aid in cell/ROI selection for barcoding, this should be done after A-tailing and before barcoding.

IMPORTANT: Do not use normal blocking serum, as it may contain enzymes that can destroy the RNA and dislodge cDNAs from the sample. Be sure to use molecular grade reagents for blocking.

IMPORTANT: If you will use this stain to visualize your ROI during barcoding (when barcode strands are hybridized to the docking sites on cDNAs within sample), **do NOT use a stain that requires UV illumination for imaging**, as imaging this stain will induce photocrosslinking of the barcodes onto cDNAs in your sample. We recommend using Red or Far-Red channels for pre-barcoding stains.

1. Make RNase-free Blocking Solution.
   RNase-free Blocking Solution for Antibody Staining (1% BSA in PBS-Tween)
   1 mL 10% (weight to volume) molecular grade recombinant BSA
   10 µL 100% Tween-20
   1 mL 10X PBS
   8 mL UltraPure water
2. Add 50 µL of Blocking Solution to each well. Incubate for 30 min to 1 hr at room temperature.
3. Add 50 µL primary antibody at desired dilution, diluted in block. Wash with **0.1% PBS-Tw** 3 x 5 minutes.
4. Add 50 µL secondary antibody at desired dilution, diluted in block. Wash 3 x 5 minutes with **0.1% PBS-Tw**.

*** DO NOT USE UV-CHANNEL FOR STAINS *!**

5. Proceed to barcoding.

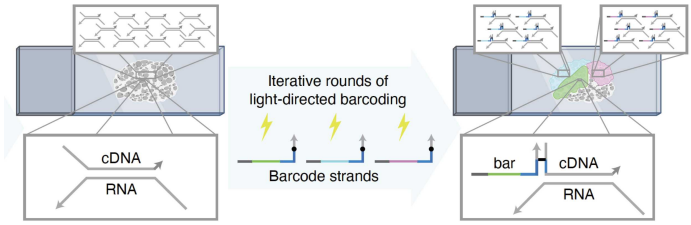
7 **Choosing an Optical System**

Before starting, ensure that your optical system is set up and calibrated properly, so that UV light can be focused on cells/regions of interest (ROIs).

Light-Directed Barcoding of Select Cells for Sequencing

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

8 Light-directed barcoding is performed sequentially for different regions of interest/cell populations within the same sample. For sequencing three regions, three rounds of barcoding are performed with a unique barcode sequence used in each round. The three barcodes used in the publication are listed in the table below, and in **Supplementary Table 6**.



The [cnvK] modification forms a crosslink to the opposing DNA strand upon hybridization to a complementary sequence and illumination with UV light (365 nm optimal). In this way, the barcoding of cDNAs within a sample can be spatially restricted using a microscope, by targeting UV illumination to regions of interest while the barcode strand is hybridized to the dock sites that were introduced on the reverse transcription primers.

Light-Seq Barcode Sequences

A	B	C	D	E
Sequence name	**Purpose**	**Sequence**	**Supplier**	**Purification**
GATE.D12.B1	Barcode sequence 1 - Cy5 labeled barcode strand.	GGAGTTGGAGTGAGTGGATGAGTGATGDDDDDDDDDDDDDTATGGATGAGTTATATAACTCA[cnvK]TCGTGTAAAT[Cy5-3]	GeneLink	PAGE
GATE.D12.B2	Barcode sequence 2 - Cy3 labeled barcode strand.	GGAGTTGGAGTGAGTGGATGAGTGATGDDDDDDDDDDDDDGTTAGGTGAGTTATATAACTCA[cnvK]TCGTGTAAAT[Cy3-3]	GeneLink	PAGE
GATE.D12.B3	Barcode sequence 3 - Fluorescein (FITC) labeled barcode strand.	GGAGTTGGAGTGAGTGGATGAGTGATGDDDDDDDDDDDDDAGGGTATGAGTTATATAACTCA[cnvK]TCGTGTAAAT[Fl-3]	GeneLink	PAGE

Note: *Fluorescently labelled barcode strands are light-sensitive and should be protected from light when possible, although some ambient light is acceptable. We routinely do all steps in well-lit rooms, but cover the samples and tubes with foil during incubation periods. A* ***Barcoding Master Mix*** *is made (with excess) on the day-of and kept at room temperature, containing all barcoding reagents with the exception of the barcode strands (GATE.D12.****B[0-2]****) and Salmon Sperm DNA.*

Barcoding Reagents

A	B	C
Reagents	**Supplier**	**Cat. No.**
Dextran Sulfate 50% Solution	Sigma Aldrich	S4030
Salmon-sperm DNA	Thermo Fisher	AM9680
5 M NaCl	Invitrogen	AM9760G
TWEEN® 20	Sigma Aldrich	P9416- 50ML
PBS	Invitrogen	AM9625
Formamide	Invitrogen	AM9342
UltraPure Water	Invitrogen	10977023
Barcode DNA Oligos (see sequences table)	GeneLink	

Light-Directed Barcoding of cDNAs

1. Prepare barcoding solutions:

Stringent 60% Wash (0.1% PBS-Tw + 60% formamide):
4 mL 10X PBS
24 mL 100% formamide
12 mL UltraPure H2O
40 µL Tween-20
Note: *Store at 4C for use later during barcoding.*

High-Salt Wash (1X PBS + 1 M NaCl + 0.1% Tween- 20):
4 mL 10X PBS
8 mL 5 M NaCl
28 mL UltraPure H2O

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

40 µL Tween-20
Note: *Store at room temperature for use later during barcoding.*

Hybridization Master Mix

A	B	C
Reagent	**Reaction concentration**	**µL per 50 µL well****
10X PBS	1x	5
5M NaCl	500 mM	5
UltraPure water		18.7
Dextran Sulfate 50% solution	10%	10
10 mg/mL sheared salmon sperm DNA	2 mg/mL	10
TWEEN® 20	0.1%	0.05
Total		48.75

** Dextran sulfate makes this mix viscous, so it is recommended to make significant excess (~1.2X what is needed for the number of wells).

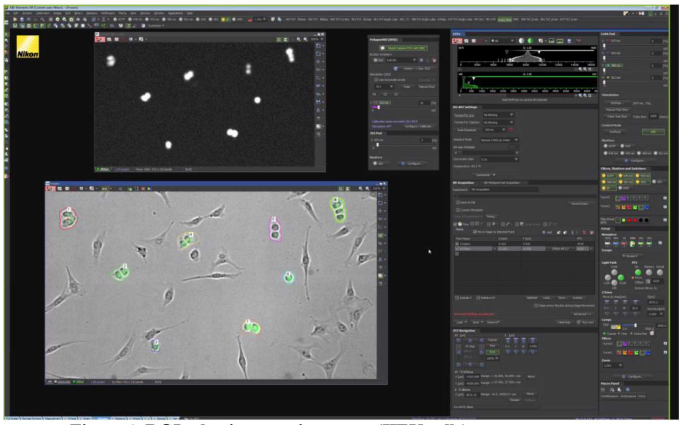
2. Add barcoding strands to **Hybridization Master Mix** to create **Barcoding Solutions**:

Barcode 0 Solution (per 50 uL well - make excess!):
1.25 µL of 10 µM Barcode Strand (**GATE.D12.B0**)
48.75 µL hybridization master mix

Barcode 1 Solution (per 50 uL well - make excess!):
1.25 µL of 10uM Barcode Strand (**GATE.D12.B1**)
48.75 µL hybridization master mix

Barcode 2 Solution (per 50 uL well - make excess!):
1.25 µL of 10uM Barcode Strand (**GATE.D12.B2**)
48.75 µL hybridization master mix

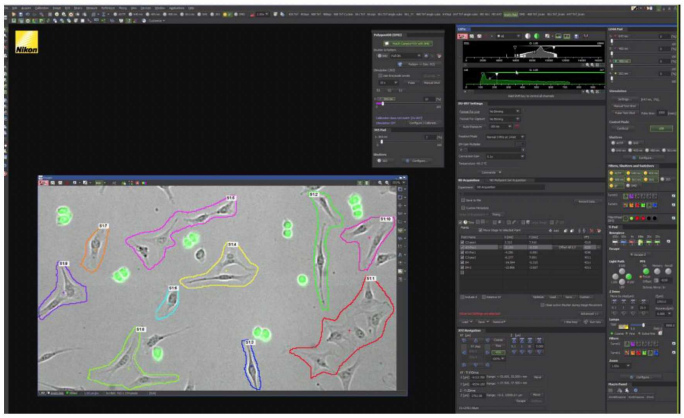
3. Replace liquid in each well with **High-Salt Wash**.
4. Aspirate to remove all liquid, add **Barcode 0 Solution.** Incubate for 30 min at room temperature to allow barcodes to hybridize to cDNA 5' overhangs. Cover slide with foil to protect from light.
5. Aspirate to remove all liquid, then wash 3 x 1 min in **High-Salt Wash.**
6. Replenish the well with **High Salt Wash**. Ensure that the sample is completely covered in liquid.
7. Bring sample to the microscope to perform imaging and light-directed barcoding. Use bright-field or histological stain to visualize cells/region of interest. Be sure to avoid using the UV channel for imaging stains, as this will induce off-target photo-crosslinking of barcodes outside of the region of interest.
Note: *If evaporation occurs during barcoding, add more* ***High Salt Wash****.*
8. Perform light-directed barcoding.



GFP+ HEK cells were co-cultured with mouse 3T3 cells and the two cell populations were barcoded in two successive rounds of barcoding. Screenshot of the Nikon Elements software with GFP image overlaid with bright field and with ROIs outlined around GFP+ cells. In this case, cells were manually outlined in the Nikon Elements Software using the Bezier ROI tool.

9. Remove slide from microscope and wash 8 times consecutively in **Stringent 60% Wash.**
Note: *For applications where very few cells are targeted (<50 cells), increasing the number of stringent washes will likely help reduce background. We recommend up to doubling the number of stringent washes after each round of barcoding.*
10. Wash 2 x 2 min in **High-Salt Wash**, then add fresh **High-Salt Wash.**
11. For additional barcoding rounds to sequence additional cell populations, repeat steps 3-10 with **Barcode 1 Solution** and **Barcode 2 Solution**.

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

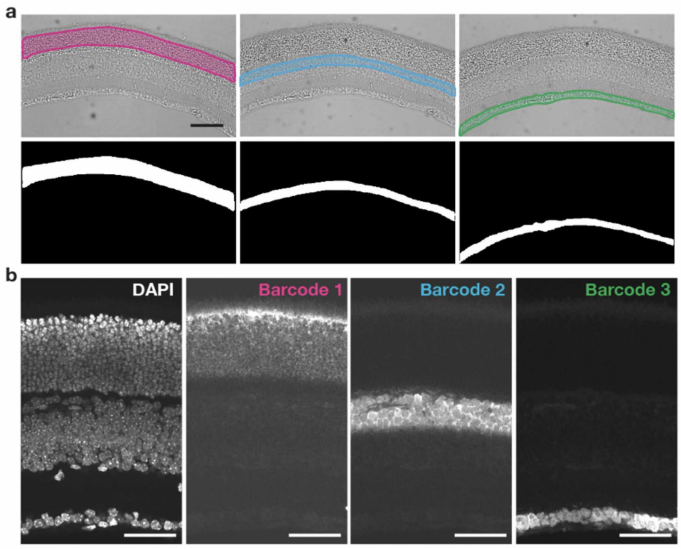


Screenshot of the Nikon Elements software with GFP image overlaid with bright field and with ROIs outlined around GFP- 3T3 cells. In this case, cells were manually outlined in the Nikon Elements Software using the Bezier ROI tool.

12. Transfer to 0.1% PBS-Tween and optionally image barcoded cDNAs to visualize.

Optional Pause Point : Store at 4C overnight in a humidified chamber. If using Grace Bio-Labs chambers, be sure to cover the holes with a coverslip or stickers to prevent evaporation.

Note: *The fluorescent barcode strands photo-bleach quickly and may appear dim even if barcoding worked well. Acquisition settings similar to those used for smFISH visualization are recommended as a starting point. At this point, DAPI and WGA can be added, and the 405 nm channel can be used for staining and imaging.*

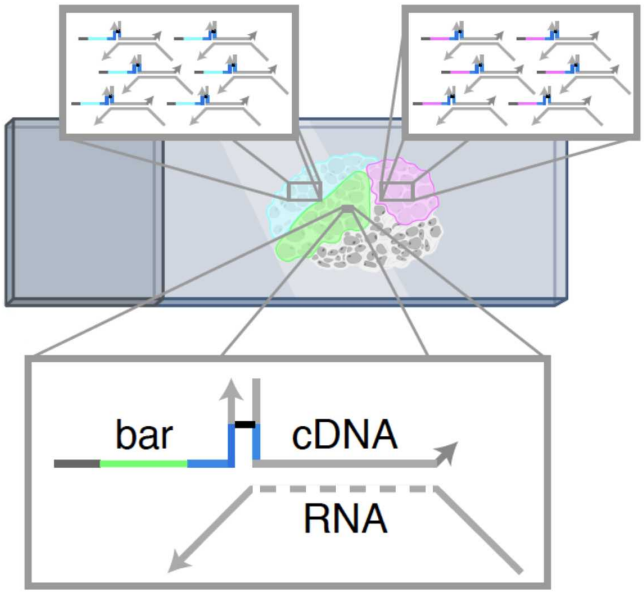


These images are from the experiment comparing transcriptomes of cellular layers in the mouse retina in the Light-Seq publication. Three distinct cellular layers in 18 um cryosections were barcoded with 3 rounds of light-directed barcoding. (a) ROIs selected for barcoding based on bright-field image were converted to a binary TIFF mask by the microscope software (Nikon Elements). (b) Post-barcoding, retinas were stained with DAPI and the fluorescent barcodes were imaged on a spinning-disk confocal microscope.

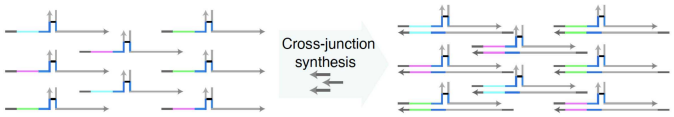
Displacement of cDNAs

9 Now that cDNAs are barcoded and A-tailed, they must be extracted for library preparation and sequencing. To do this, the RNA template is digested by mild RNaseH treatment, which degrades the RNA bound to the barcoded cDNAs *in situ*, liberating the barcoded cDNAs for collection.

Note: *Use low retention tips.*



The displaced barcoded-cDNAs contain a photocrosslink, preventing normal amplification by PCR. Therefore, the Cross-Junction Synthesis reaction creates a single stitched product strand containing the DNA barcode and the cDNA sequence, producing a single amplifiable strand for library preparation and sequencing.



Displacement Reagents

A	B	C
Reagents	**Supplier**	**Cat. No.**
ThermoPol® Reaction Buffer Pack	NEB	B9004S
RNase H - 1,250 units	NEB	M0297L
UltraPure Water	Invitrogen	10977023

Cross-Junction Synthesis Reagents

A	B	C
Reagents	**Supplier**	**Cat. No.**
Bst DNA Polymerase, Large Fragment- 8,000 units	NEB	M0275L
ThermoPol® Reaction Buffer Pack	NEB	B9004S
UltraPure Water	Invitrogen	10977023
Deoxynucleotide (dNTP) Solution Mix - 8 µmol at 10mM each	NEB	N0447S

Cross-Junction Synthesis Primer

A	B	C	D	E
Name	Description	Sequence	Supplier	Purification
GATC.20T.p	Primer for Cross-Junction Synthesis	GAGAATGTGAGTGAAGATGTATGGTGATTTTTTTTTTTTTTTTTTT	IDT	HPLC

Displace and collect cDNAs separately for each well

1. Dilute cross-junction synthesis primer GATC.20T.p in UltraPure water to make 1 uM stock.
   5 µL 10 µM primer (**GATC.20T.p**)
   45 µL UltraPure water
2. Make **Displacement Mix.**

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

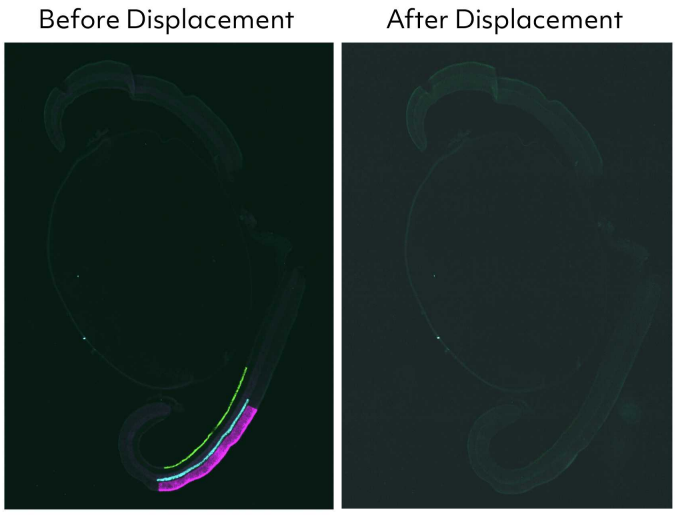
A	B	C
Reagent	**Reaction concentration**	**uL reagent per 50 uL well**
10x ThermoPol Reaction Buffer	1x	5 µL
RNase H (from NEB) (5,000U/mL)	250 U/mL	2.5 µL
UltraPure Water		42.5 µL
Total		**50**

3. Aspirate liquid from wells and add **50 µL** of **Displacement Mix** to each well.
4. Cover the well holes with a drop of Mineral Oil and incubate at 37C for 45min.

NOTE: *If using Grace Bio-Labs chambers, we recommend Mineral Oil to minimize loss of solution during displacement.*

5. While samples are incubating, prepare a separate collection tube for each well by adding **1.2 µL of 1 µM primer** (**GATC.20T.p**) to a low bind PCR tube.
6. After the incubation, pipette well solution up and down 5-7 times in the well without creating bubbles.
7. Collect **all liquid** from the well in the corresponding collection tube, prepared in Step 5. Repeat for all wells using fresh tips for each.
8. Wash wells 3 times with **0.1% PBS-Tw**. Move to 4C in a humidified chamber for storage, ensuring the wells are covered to prevent evaporation.
9. Heat inactivate the collection tubes in a thermocycler at 75C for 20min.
10. Move tubes to ice and proceed to **Cross-Junction Synthesis**.

Optional: Samples can be imaged after displacement to verify that the fluorescent barcode signal is no longer present. At this point, follow-up stains can be performed on the sample including antibody stains, H&E, etc.



Comparison of tissue before and after displacement. The fluorescent barcode strands should be largely removed upon displacement. In some tissues/cases, the displacement may not be this efficient. It is possible to get some fluorescent barcode strands stuck within the sample.

Cross-Junction Synthesis

1. Add 8.16 µL **Cross-Junction Synthesis Mix** to each tube:

A	B	C
Reagent	**Final Reaction concentration**	**uL reagent per 50 uL well**
10X ThermoPol buffer	1x	0.92
10mM dNTPs	100 µM	0.59
Ultrapure water		0.85
Bst LF polymerase (8,000U/mL)	800U/mL	5.8
Total		**8.16**

Note: These volumes are for 50 uL wells, and volumes should be scaled according to well volume. Note that the primer (**GATC**.20T.p) was added in the previous step, before heat inactivation.

2. Vortex and spin briefly.
3. Incubate in a thermocycler for:
   37C for 30 mins
   80C for 20 mins
4. The resulting products are **CJS Samples**. Proceed to qPCR to amplify **CJS Samples**.

Optional Pause Point : Store at -20C overnight.

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

qPCR Amplification

10 Cross-Junction Synthesis libraries of barcoded cDNAs are now amplified and prepared for sequencing. We use the Kapa Hifi HotStart polymerase. We recommend performing qPCR with 30 cycles on a small subset of each sample (5 uL sample in a 10 uL PCR), to identify the appropriate cycle number for optimal amplification. 2h

qPCR Primers

A	B	C	D	E
Name	**Description**	**Sequence**	**Supplier**	**Purification**
GATE	PCR Primer 1	GGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
GATC	PCR Primer 2	GAGAATGTGAGTGAAGATGTATGGTGA	IDT	HPLC

Reagents for qPCR

A	B	C
Reagents	**Supplier**	**Cat. No.**
SYBR™ Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO	Invitrogen	S7563
HiFi HotStart DNA Polymerase, KapaBiosystems	Roche	KK2502
UltraPure Water	Invitrogen	10977023

qPCR to Determine Amplification Cycle Number

1. Vortex **CJS Samples**.
2. Dilute Sybr Green I dye in water to make 5x solution (e.g. dilute 10,000X stock twice, 1:100 in water, then 1:20 in water).
3. Create PCR master mix:

A	B
Kapa qPCR Master Mix	
Reagent	µL reagent per 10 µL reaction
5x SYBR Green I	1
Kapa HiFi HotStart buffer (5x)	2
10 µM GATE primer	0.3
10 µM GATC primer	0.3
10 mM dNTPs from Kapa kit	0.3
UltraPure water	0.9
Kapa HiFi Hot Start polymerase	0.2
Total	5

The ratio of Kapa qPCR Master Mix to Cross-Junction Synthesis Solution is 1:1, so each sample will have a 10 uL total PCR with 5 uL of Kapa qPCR Master Mix and 5 uL of Cross-Junction Synthesis Solution.

4. Quickly vortex and spin down master mix, then add 5 uL master mix into each tube.
5. Add 5 uL of appropriate sample from Cross-Junction Synthesis reaction to each tube.
6. Quickly vortex and spin down reactions.
7. Place into qPCR machine for 30 cycles and run the **qPCR Machine Protocol.**

qPCR Machine Protocol
98C for 3 minutes
30 cycles of:
98C for 20 seconds
60C for 30 seconds
72C for 2 minutes
Plate read
72C for 5 minutes
Melting curve analysis
Hold at 4C

8. Check amplification graph to choose appropriate cycle number.

NOTE: *Cycle number for amplification of the remaining Cross-Junction Synthesis Product should be chosen to prevent over-amplification of the library. In general, the optimal cycle number will correlate with the size of the barcoded region, which directly impacts how much amplifiable cDNA exists within the reaction. Note that for technical replicates, the amplification curves are generally very consistent. In general, optimal cycle number will vary across experiments and we recommend that the full PCR is run at the optimal cycle number per experiment.*

11 After the test qPCR on a small amount of sample, a PCR is performed on all of the **CJS Samples** to amplify the entire sample for library preparation and sequencing. The cycle number for amplification is chosen based on the test qPCR in the previous step, to prevent over-amplification. Each Cross-Junction Synthesis product (1 per sample well) is amplified in a separate tube. This protocol is identical to the prior qPCR, but scaled to amplify the entire sample 2h

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

and with a reduced number of cycles.

All of the sample should be amplified. However, most qPCR machines have a limit of 50 uL/reaction. Therefore, the full volume should be split into multiple PCRs to ensure accurate amplification.

Amplification of Full Samples

1. Briefly vortex and spin **CJS Samples**.
2. Measure the volume of CJS sample in each tube and use to calculate volume of qPCR master mix needed for each sample (**1:1 ratio of qPCR master mix : CJS sample**).

Note: *Ensure that PCR volumes do not exceed the limits for your machine and otherwise, split into multiple tubes.*

3. Dilute Sybr Green I dye in water to make 5x solution (e.g. dilute 10,000X stock twice, 1:100 in water, then 1:20 in water).
4. **Create qPCR master mix:**

A	B
Kapa Full qPCR Master Mix	
Reagent	**µL reagent per 50 µL reaction**
5x SYBR Green I	10
Kapa HiFi HotStart buffer (5x)	20
10 µM GATE primer	3
10 µM GATC primer	3
10 mM dNTPs from Kapa kit	3
Ultrapure water	9
Kapa HiFi hot start polymerase	2
Total	**50**

The ratio of Kapa qPCR Master Mix to Cross-Junction Synthesis Solution is 1:1, so each sample will have a 10 µL total PCR with X µL of Kapa qPCR Master Mix and X µL of Cross-Junction Synthesis Solution.

5. Add the entire remaining sample with equal parts master mix to each tube.
6. Quickly vortex and spin down reactions.
7. Place into qPCR machine for exactly **XX** cycles and run the **Full qPCR Machine Thermocycler Program.**

Full qPCR Machine Thermocycler Program

98C for 3 minutes
XX cycles of:
98C for 20 seconds
60C for 30 seconds
72C for 2 minutes
Plate read
72C for 5 minutes
Hold at 4C

***XX is based on the amplification curves from the test qPCR. This number will likely range between 16 and 25.**

8. The resulting products are **Amplified Samples.**

Library Preparation for Illumina Sequencing 5h

12 Light-Seq uses conventional tagmentation-based library preparation for Illumina sequencing, but with custom primers for the secondary PCR and for Read 1/i5 sequencing. The custom primers are necessary to specifically enrich for and sequence amplicons containing the light-directed barcode sequences. The first step is bead purification of the amplified libraries for each well, followed by tagmentation, secondary PCR amplification, and a second bead purification.

Library Preparation Reagents

A	B	C
Reagents	**Supplier**	**Cat. No.**
Magnetic Separator (or equivalent)	10X Genomics	120250
Ampure XP Beads	Beckman	A63881
Ethyl alcohol, pure (200 proof)	Sigma Aldrich	E7023-1L
Qubit™ 1X dsDNA High Sensitivity (HS) and Broad Range (BR) Assay Kits	Invitrogen	Q33231
Nextera XT Library Preparation Kit	Illumina	FC-131-1096
UltraPure Water	Invitrogen	10977023

Library Preparation DNA Oligos

These primer sequences are used for unique indexing of samples for pooled sequencing. For each sample, a unique pair of S50X and Next.N70X are required. You do not need to order all primers, only enough pairs to uniquely index your samples of interest. Because the custom i5 index primer does not work well on all Illumina machines, we highly recommend each sample be prepared with a unique i7 index. We hope to adjust our recommendations on this front soon, so stay tuned for updated protocols.

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

A	B	C	D	E
Name	**Description**	**Sequence**	**Supplier**	**Purification**
S502.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACCTCTCTATCGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
S503.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACTATCCTCTCGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
S505.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGCGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
S506.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACACTGCATACGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
S507.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACAAGGAGTACGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
S508.GATE	Primer for library prep - i5 (barcode) side.	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTCGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
Next.N701	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG	IDT	HPLC
Next.N702	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG	IDT	HPLC
Next.N703	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG	IDT	HPLC
Next.N704	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG	IDT	HPLC
Next.N705	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG	IDT	HPLC
Next.N706	(Normal) primer for library prep - i7 side.	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG	IDT	HPLC

Bead purification of Amplified Samples

NOTE: *Each well/replicate is processed separately* .

1. Make 50 mL of 80% Ethanol, diluted in UltraPure water.
2. For each well, combine **80 µL Amplified Sample** with **96 µL Ampure XP Beads** in a new PCR tube**.**
3. Mix well by pipetting (incubate at room temperature for **5 min**). DO NOT overextend this step.
4. Place on Magnetic Separator Stand for 2 min.
5. Discard supernatant. Wash 3 x 30 sec with 200 µL of 80% Ethanol.
6. Discard ethanol and aspirate remainder with a small 10 µL tip.
7. Air dry for ~5 min, until beads are dry. Beads will change color slightly when dry.
8. Add 30 µL of water to resuspend the beads.
9. Remove from the magnetic stand. Mix well by pipetting or vortexing and incubate for 2 min at room temperature.
10. Place Magnetic Separator Stand for 1 min. **Collect supernatants** in new PCR tubes. These are **Purified Amplified Samples.**

Measure DNA concentrations of Amplified Samples with Qubit HS dsDNA assay

This should be done according to the manufacturer's instructions, found here: https://assets.fishersci.com/TFS-Assets/LSG/manuals/MAN0017455_Qubit_1X_dsDNA_HS_Assay_Kit_UG.pdf.

1. Measure concentrations using Qubit Fluorometer.
2. Record concentrations of each **Purified Amplified Sample**.

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1

Perform Nextera tagmentation

Reagents in this section are from the Nextera XT Library Preparation Kit (Illumina cat. no. FC-131-1096). Tagmentation will fragment the amplified PCR products into shorter sequences, and the length of the reaction time determines how much fragmentation occurs. Therefore, it is very important to follow the times strictly.

1. In a clean set of PCR tubes, aliquot 7 µL of Neutralize Tagment Buffer (NT). This will be used to stop the tagmentation and prevent over-tagmentation. One tube per sample/well is required. Set aside, next to the thermocycler.
2. To a new PCR tube for each Purified **Amplified Sample** (on ice):
   a. Add 10 µL of tagment DNA buffer (TD)
   b. Add 2 ng of purified **Purified Amplified Samples** and add water up to 5uL. Calculated sample and water volume to add based on the Qubit concentrations from previous step.
   c. Add 5 µL of Amplicon Tagment Mix (ATM) to lid of tube. This contains the enzyme.
3. Briefly spin down tubes, vortex, and spin down.
4. Place into PCR machine and incubate at **55°C for EXACTLY 5 minutes**.
5. Immediately stop reactions with 5 µL of Neutralize Tagment buffer (NT) (use a multichannel to mix).
6. Vortex and spin to ensure full mixing. Hold **Tagmented Samples** on ice.

Sample indexing PCR with unique i5 and i7 primer pairs

To pool **Tagmented Samples** from each well/replicate for sequencing, each sample is assigned a unique pair of i7 and i5 primer sequences. The primer pairings should be decided and documented. Each **Tagmented Sample** will be amplified briefly in this step to attached the sample-specific i5 and i7 sequences.

1. Assign and record unique index primer pairs to each sample. For example:

A	B	C
Sample	**i5 primer**	**i7 primer**
Tagmented Sample 1 (Well 1)	S502.GATE	Next.N701
Tagmented Sample 2 (Well 2)	S503.GATE	Next.N702
Tagmented Sample 3 (Well 3)	S505.GATE	Next.N703
Tagmented Sample 4 (Well 4)	S506.GATE	Next.N704

Each sample will receive a different pair of primers in the indexing PCR.

2. To each **Tagmented Sample** tube from the previous tagmentation step:
   a. Add 6.5 µL water
   b. Add 1.75 µL of standard Nextera (i7: **Next.N70X**) primer (from 10 uM stock)
   c. Add 1.75 µL of custom Nextera (GATE, i5: **S50X.GATE**) primer (from 10 uM stock)
   d. Add 15 µL PCR mix (NPM PCR master mix)
3. Vortex and spin down reactions.
4. Place in thermocycler and start **Indexing PCR Program**.

Indexing PCR Program

72°C for 3 minutes
95°C for 15 seconds
12 cycles of:
95°C for 15 seconds
55°C for 15 seconds
72°C for 40 seconds
72°C for 1 minute
Hold at 10°C

5. The resulting tubes are the **Indexed Samples.**

Purify indexed samples with Ampure Beads

1. Make 50 mL of 80% Ethanol, diluted in UltraPure water.
2. For each well, combine 50 µL of **Indexed Sample** and mix with 45 µL **Ampure XP Beads** in a new PCR tube.
3. Mix well by pipetting (incubate at room temperature for **5 min**). DO NOT overextend this step.
4. Place on Magnetic Separator Stand for 2 min.
5. Discard supernatant. Wash 3 x 30 sec with 200 µL of 80% Ethanol.
6. Discard ethanol and aspirate remainder with a small 10 µL tip.
7. Air dry for ~5 min, until beads are dry. Beads will change color slightly when dry.
8. Add 30 µL of water to resuspend the beads.
9. Remove from the magnetic stand. Mix well by pipetting or vortexing and incubate for 2 min at room temperature.
10. Place on Magnetic Separator Stand for 1 min.
11. Collect supernatants in new tubes. These are the **Purified Indexed Samples.**

Measure DNA concentrations and length of Indexed Samples

1. Measure concentrations using Qubit HS dsDNA assay. This should be done according to the manufacturer's

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instructions, found here: https://assets.fishersci.com/TFS-Assets/LSG/manuals/MAN0017455_Qubit_1X_dsDNA_HS_Assay_Kit_UG.pdf.
2. Record concentrations of each **Purified Indexed Sample**.
3. Run 2 uL of each **Purified Indexed Sample** on a 1% agarose gel.

Sequencing

13 This library preparation is compatible with standard Illumina next-generation sequencing. Note that custom Read 1 and i5 index primers are required and are listed in the table below.

DNA Oligos for Sequencing

A	B	C	D
Description	**Sequence**	**Supplier**	**Purification**
Custom Read 1 Primer -required for sequencing ofamplicons.	CGCCGGAGTTGGAGTGAGTGGATGAGTGATG	IDT	HPLC
Custom i5 index primer -required for some Illuminasequencers (see caption).	CATCACTCATCCACTCACTCCAACTCCGGCG	IDT	HPLC

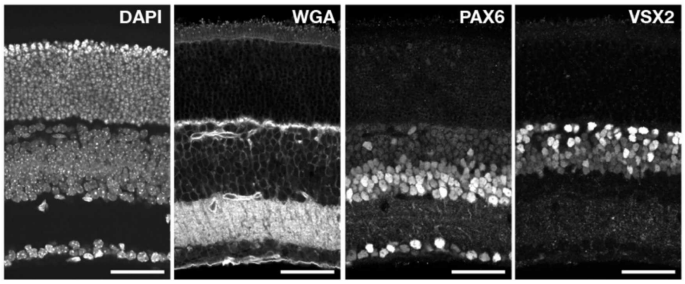
We note that the custom index primer is compatible with HiSeq, but for NovaSeq, unique i7 indices were needed for de-convolution due to inefficient i5 index sequencing.

We have found adding 60% above the standard concentration of each custom primer works well on many of the Illumina sequencers and would recommend this as a starting point.

We have had good success with elevated custom primer concentrations on Illumina MiSeq and NextSeq machines. The standard custom primer concentrations worked for HiSeq. For NovaSeq, we haven't seen as good efficiency even with the elevated custom primer concentrations, particularly for the custom i5 index sequencing but hope to have updated recommendations soon. For now, we recommend having unique i7 index sequences for each sample to aid in sequence de-multiplexing, and that you check back here periodically or reach out for updated sequencer-specific recommendations.

Post-Sequencing Tissue Staining

14 Tissue can stored for 2+ weeks in 1X PBS and can be stained after sequencing. You should cover the sample appropriately to ensure that it does not dry out. We recommend also using a hybridization chamber, and replensihing the liquid in the wells every few days. While some antigens might be disrupted by the protocol, we have detected proteins via immunofluorescence with success in addition to other stains.



Stains for genomic DNA (DAPI), cell membranes (WGA), and two retinal proteins (PAX6 and VSX2) were detected after sequencing. This image was from one of the tissue sections sequenced in the cellular layers experiment from the Light-Seq paper.

Citation: Jocelyn Y. Kishi, Ninning Liu, Emma R. West, Kuanwei Sheng, Jack J. Jordanides, Matthew Serrata, Constance L. Cepko, Sinem K. Saka, and Peng Yin Light-Seq https://dx.doi.org/10.17504/protocols.io.x54v9jno4g3e/v1