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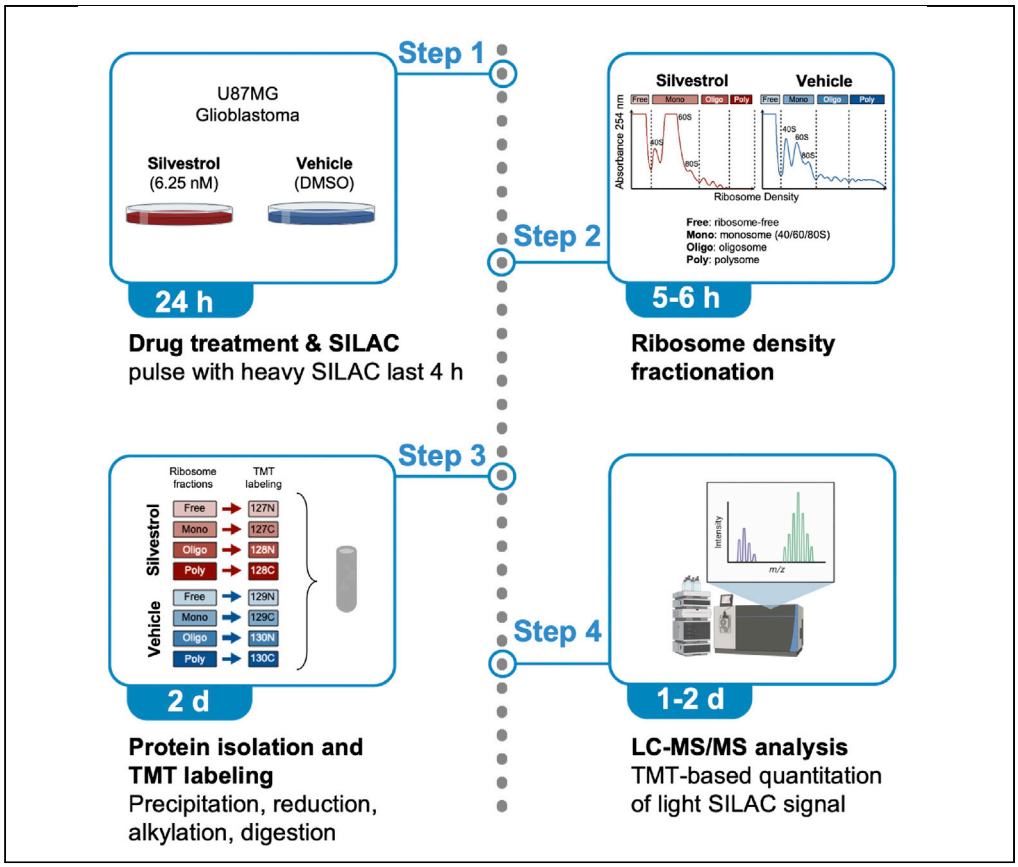
MATRIX platform to analyze translation machinery remodeling in glioblastoma cells



Here, we present a protocol using MATRIX (mass spectrometry analysis of active translation factors using ribosome density fractionation and isotopic labeling experiments) platform to investigate changes of the protein synthesis machinery in U87MG glioblastoma cells in response to the rocaglate silvestrol. This protocol describes steps to perform SILAC (stable isotope labeling by amino acids in cell culture), ribosome density fractionation, protein isolation, and mass spectrometry analysis. This approach can be applied to study any adaptive remodeling of protein synthesis machineries.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A platform to analyze translation machinery remodeling in glioblastoma cells

MATRIX protocol isolates the translation machinery from newly synthesized peptides

Combines SILAC, ribosome density fractionation, and LC-MS/MS to assess response to silvestrol

MATRIX is adaptable to a variety of stress conditions such as hypoxia

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MATRIX platform to analyze translation machinery remodeling in glioblastoma cells

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SUMMARY

Here, we present a protocol using MATRIX (mass spectrometry analysis of active translation factors using ribosome density fractionation and isotopic labeling experiments) platform to investigate changes of the protein synthesis machinery in U87MG glioblastoma cells in response to the rocaglate silvestrol. This protocol describes steps to perform SILAC (stable isotope labeling by amino acids in cell culture), ribosome density fractionation, protein isolation, and mass spectrometry analysis. This approach can be applied to study any adaptive remodeling of protein synthesis machineries.

For complete details on the use and execution of this protocol, please refer to Ho et al. (2021).[1]

BEFORE YOU BEGIN

The following protocol describes the specific steps for using U87MG glioblastoma cells with silvestrol but can be adapted to other cell types and treatment conditions. At the time this experiment was conducted, the rocaglate clinical candidate zotatifin (NCT04092673) was not commercially available. Contrary to other mechanistic studies of rocaglates, we used silvestrol at a lower dose and longer duration to study a more biologically relevant tumor response to treatment. Use of 6.25 nM silvestrol over 24 h enabled analysis of eIF4A1 inhibition without the confounding effects of overwhelming apoptosis and corresponded to the plasma steady-state concentration in rats. The workflow of MATRIX is detailed in Figure 1A. Polysome absorbance profiles from ribosome fractionation steps are shown in Figure 1B.

1. Cells should be cultured in light (R_0K_0) SILAC media for 7 d prior to start of a drug treatment or other manipulation.
2. Prepare buffers prior to starting. Cycloheximide (CHX) and sucrose are dissolved in polysome basic solution.
3. For drugs like silvestrol that are resuspended in DMSO, we limit cytotoxicity by restricting final DMSO concentration to 0.1%.

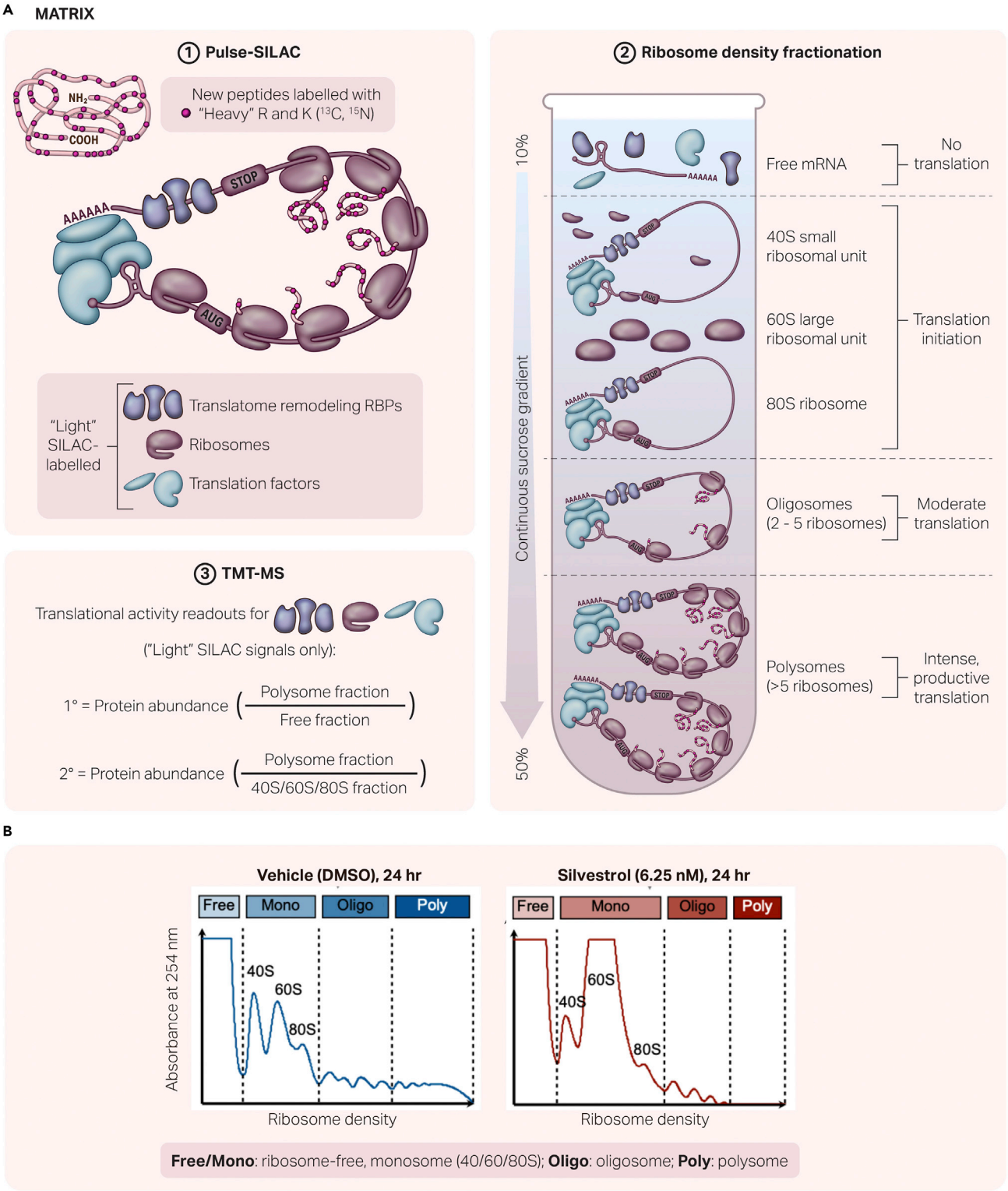

Figure 1. Workflow of MATRIX analysis

(A) 1. Pulse-SILAC heavy labeling of newly-synthesized proteins to be excluded from analysis. 2. Ribosome density fractionation enables separation of mRNAs based on number of bound ribosomes and subunits. mRNAs in the polysome fraction (>5 ribosomes) represent intense protein translation of their transcripts. 3. TMT-MS enables identification of proteins associated with the ribosome fractions. We exclude the confounding of newly-synthesized proteins by only including the light SILAC signals.

(B) Representative ribosome fractionation absorbance profiles for the experiment outlined in this protocol.

4. U87MG cells are grown in 15-cm^2 dishes and maintained at 37°C in a 5% CO_2 humidified incubator. One to two 15-cm^2 plates are needed for fractionation for each condition.
5. Fractionation system should be baselined before the experiment.

Sucrose gradients

Timing: 6–7 h

Sucrose gradients are used to separate cellular mRNA based on the number of ribosomes bound to the transcript. This step describes the preparation of these gradients for downstream use.

Note: Prepare gradients the day before you plan on harvesting cells (d-1).

Note: Cycloheximide (10 mg/mL in polysome basic solution) should be freshly prepared.

Note: For preparation of sucrose gradients, use a 10 mL syringe and long needle to transfer the solutions into the ultracentrifuge tubes. Keep tubes in a rack for step 1 to minimize disturbances to the sucrose layers.

6. Prepare 10% (w/v) sucrose and 50% (w/v) sucrose solutions each with 100 μg/mL CHX.
   a. Transfer 5.2 mL 10% sucrose into ultracentrifuge tube.
   b. Slowly transfer 5.2 mL 50% sucrose into bottom of tube, beneath the 10% layer.
   c. Carefully cover each tube with parafilm.

⚠ CRITICAL: Carefully handle the gradient tubes, avoiding unnecessary disturbances and mixing.

Note: 10% and 50% layers should be level and matching the other tubes. There should be equal volumes in each tube, and the meniscus separating the two sucrose layers should be the same height across tubes.

7. To generate linear gradients, lie the tubes horizontally at 4°C for 5–6 h.
   a. Gently transfer the tube in an upright (vertical) position from the rack to a solid, flat surface at 4°C (shelf of fridge, benchtop in cold room).
   b. With the tube upright, set the base of the tube on the surface.
   c. Keeping the base fixed, slowly move the top of the tube toward you until the tube is lying completely flat; the tube is now in the horizontal position.
   d. Ensure that the tubes do not roll or are disturbed and leave horizontal at 4°C for 5–6 h.
   e. To move the tubes back to the vertical position, keep the base of the tube fixed and gently lift the top of the tube up and away from you.
   f. Carefully transfer tubes to a rack and store at 4°C.

Alternatives: Commercially available linear gradient makers may be used instead.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Silvestrol	MedChemExpress	Cat#HY-13251
Cycloheximide	MilliporeSigma	Cat#01810

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
RNAseOUT Recombinant Ribonuclease Inhibitor	Thermo Fisher	Cat#10777019
Triton X-100	MilliporeSigma	Cat#T8787
NaCl	MilliporeSigma	Cat#S9625
$MgCl_2 \cdot 6H_2O$	MilliporeSigma	Cat#M2670
Tris-HCl	MilliporeSigma	Cat#10812846001
Sucrose	MilliporeSigma	Cat#S7903
D-PBS	Thermo Fisher	Cat#14190144
DMEM	Corning	Cat#10-013-CV
Fetal bovine serum	Genesee Scientific	Cat#25-514
Penicillin/streptomycin	Cytiva	Cat#SV30010
L-Arginine-HCl for SILAC	Thermo Fisher	Cat#88434
L-Leucine for SILAC	Thermo Fisher	Cat#88428
Iodoacetamide	MilliporeSigma	Cat#I1149
Urea	MilliporeSigma	Cat#U1250
NH_4HCO_3 (pH 8.3)	MilliporeSigma	Cat# A6141
DTT	MilliporeSigma	Cat#43815
Trypsin/LysC	Promega	Cat#V5072
Trifluoracetic acid (TFA)	MilliporeSigma	Cat#302031
Critical commercial assays		
SILAC Protein Quantitation Kit (Trypsin), DMEM	Thermo Fisher	Cat#A33972
TMT10plex™ Isobaric Label Reagent Set	Thermo Fisher Scientific	Cat#90110
Deposited data		
MATRIX mass spectrometry data (Ho et al.[1]) https://doi.org/10.1016/j.celrep.2021.109806	PRIDE via ProteomeXchange	PXD022556
MATRIX mass spectrometry data (Ho et al.[2]) https://doi.org/10.1038/s41467-020-16504-1	PRIDE via ProteomeXchange	PXD011979, PXD006799
RNA sequencing data (Ho et al.[2]) https://doi.org/10.1038/s41467-020-16504-1	NCBI GEO	GSE128541, GSE128547, GSE128555
MATRIX mass spectrometry data (Balukoff et al.[3]) https://doi.org/10.1038/s41467-020-19602-2	PRIDE via ProteomeXchange	PXD015643, PXD006799
MATRIX mass spectrometry data (Ho et al.,[4]) https://doi.org/10.1016/j.celrep.2017.12.031	PRIDE via ProteomeXchangef	PXD006799
RNA sequencing data (Ho et al., 2018)	NCBI SRA	SRP110475
Experimental models: Cell lines		
Human U87MG cell line	American Type Culture Collection	Cat#HTB-14
Software and algorithms		
PEAKS X+ (v10.5)	Bioinformatics Solutions Inc. in Waterloo, Ontario, Canada.	https://www.bioinfor.com/download-peaks-studio/
Other		
13.2-mL Open-Top Thinwall Tube	Beckman Coulter	Cat#344039
Optima L-90K Ultracentrifuge	Beckman Coulter	N/A
SW 41 Ti Swinging-Bucket Rotor	Beckman Coulter	N/A
BR-188 Density Gradient Fractionation System	Brandel	N/A
Savant SPD2010 SpeedVac concentrator	Thermo Fisher	Cat#SPD2030-220
Thermo Scientific Orbitrap Fusion-Lumos Tribid Mass Spectrometer (CA)	Thermo Fisher	N/A
Nanospray source and Dionix Ultimate 1000 nano-LC system	Thermo Fisher	N/A
PEPMAP100 C18 5 μM trap column	Thermo Fisher	N/A
PEPMAP C18 2 μM 15 cm column	Thermo Fisher	N/A

MATERIALS AND EQUIPMENT

Polysome Basic Solution		
Reagent	Final concentration	Amount
NaCl	0.3 M	8.75 g
$MgCl_2 \cdot 6H_2O$	15 mM	1.52 g
Tris-HCl, pH 7.4	15 mM	0.908 g
ddH_2O	N/A	up to 500 mL
Total	**N/A**	**500 mL**
Store at 4°C for up to 3 months.		

Polysome Lysis Buffer		
Reagent	Final concentration	Amount
Polysome Basic Solution		9.765 mL
Triton X-100	1%	100 µL
Cycloheximide 10 mg/mL	100 µg/mL	100 µL
RNAse Out	100 U/mL	35 µL
Total	**N/A**	**10 mL**
Make fresh the day of use.		

Reduction Buffer		
Reagent	Final concentration	Amount
NH_4HCO_3 pH 8.3	50 mM	39.5 mg
Urea	8 M	4.80 g
DTT	10 mM	15.4 mg
ddH_2O		Up to 10 mL
Make fresh the day of use.		

HPLC Fractionation Buffers	
Reagent	Ingredient
Buffer A	ddH_2O adjusted to pH 10 with ammonium hydroxide
Buffer B	80% acetonitrile adjusted to pH 10 with ammonium hydroxide
Make fresh the day of use.	

STEP-BY-STEP METHOD DETAILS

Drug treatment and pulse SILAC – Day 0

Timing: 24 h

Cells are treated with the rocaglate silvestrol and pulsed with SILAC media to label the newly synthesized peptides. These peptides do not represent the active translation machinery and will be discarded in downstream analysis. All media has a base of DMEM with 10% FBS and 1% penicillin-streptomycin. U87MG cells are cultured and expanded in 15-cm^2 plates in light (R_0K_0) media for 7 d prior to the start of drug treatment. Two 15-cm^2 plates each containing approximately 10 million cells (roughly 85% confluent) are used for each condition/replicate. Cells are treated for a total of 24 h with either 6.25 nM silvestrol or 1:1000 DMSO (vehicle). Our analysis was optimized for the conditions described. Shorter treatment durations and other experimental design changes would require optimization of SILAC conditions.

1. Treat cells with drug or vehicle resuspended in light (R_0K_0) SILAC media for 20 h.

Note: We used the SILAC Protein Quantitation Kit (Trypsin), DMEM from Thermo Scientific. See manufacturer's manual (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0016245_2161996_PierceSILAC_Pr..._Kits_UG.pdf) for more information about the SILAC media.

Note: Resuspend silvestrol or vehicle, same concentrations as above, in heavy ($R_{10}K_8$) media for step 3 and warm to 37°C just prior to the media exchange.

2. Wash cells once with warm (37°C) PBS.
3. Pulse cells with heavy ($R_{10}K_8$) SILAC media containing drug for last 4 h.
   a. Aspirate PBS, add prewarmed drug-containing (or vehicle) heavy ($R_{10}K_8$) SILAC media to corresponding plates.
   b. Incubate at 37°C for remaining 4 h.

⚠ CRITICAL: Due to physiologic responses to changes in temperature, it is imperative that media and PBS is prewarmed and that steps 2 and 3 are performed quickly to minimize temperature disturbances.

Ribosome density profiling (fractionation) – Day 0

Timing: 5–6 h

Ribosome density fractionation enables the separation of mRNAs based on the number of ribosomes bound to a transcript. A transcript with no ribosomes or subunits bound will shift to the top of the sucrose gradient upon centrifugation, while a transcript undergoing active translation, with multiple ribosomes bound, will shift to the bottom of the gradient. The following procedure is necessary to differentiate proteins in the sample lysate based on their engagement with translation.

Note: Prepare polysome lysis buffer fresh the day of harvest. Samples should be kept at 4°C throughout.

4. Treat cells with CHX and harvest.
   a. Treat cells with 100 μg/mL CHX for 10 min at 37°C.
   b. Wash cells two times with cold PBS containing CHX (100 μg/mL) and aspirate.
   c. Add 5 mL cold PBS containing CHX to each 15-cm plate. Harvest cells by scraping and combine the two 15-cm^2 plates into a single 15 mL tube.
   d. Centrifuge cells 100 × *g* for 5 min.
5. Lyse cell pellets.
   a. Resuspend each cell pellet in 500 μL polysome lysis buffer and transfer to a 2 mL microcentrifuge tube.
   b. Rotate tubes at 4°C for 20 min.
   c. Centrifuge tubes at 10,000 × *g* for 10 min, 4°C.
   d. Transfer supernatant to a new 2 mL microcentrifuge tube and centrifuge at 10,000 × *g* for 10 min, 4°C.
   e. Transfer supernatant to a new 2 mL microcentrifuge tube and label. Keep samples on ice.

⚠ CRITICAL: Lysates should be centrifuged twice as described above. Membranous debris is inevitably included in the lysate after only one centrifugation.

6. Quantify estimated RNA and calculate volumes for equal RNA across samples.
   a. Approximate RNA concentration of sample lysates by measuring the absorbance at 260 nm.

Table 1. Example of RNA normalization for sample loading volumes

Sample	RNA concentration (μg/μL)	Volume (μL)	Total RNA (μg)	Calculated loading volume normalized RNA (μL)
A	1.110	500	555,000	(500)
B	1.273		555,000	436
C	1.348		555,000	412
D	1.209		555,000	459

b. Calculate volumes needed for equal amounts of RNA amongst samples. See note below.

Note: A260 measurement in these lysates represents RNA in addition to free ATP, GTP, dNTPs, and DNA. Because these non-RNA components will be discarded through downstream processing, we use A260 as an approximate measurement of RNA concentration in these samples.

Note: Lysate RNA concentrations for two 15-cm^2 plates normally range from 1–2 μg/μL. We calculate total RNA for 500 μL of the sample with lowest RNA concentration. Divide this total RNA value by other sample concentrations to obtain the loading volume of lysate needed for that sample. An example is shown in Table 1 below. Sample A has the lowest RNA concentration. We calculate the total RNA in 500 μL of sample A (555,000 μg). This sets the upper limit of loading volume to 500 μL. We then determine the loading volumes for samples B, C, and D by dividing the calculated total RNA by their RNA concentrations.

7. Load sucrose gradients and balance rotor buckets.
   a. Place a rotor bucket into a rack and place on a scale. Include the cap, unattached.
   b. Carefully place the sucrose gradient centrifuge tube into the rotor bucket.
   c. Load each gradient with sample lysate by placing pipette tip on inside wall of tube and gently dispensing.
   d. Balance corresponding rotor buckets by adding polysome lysis buffer as needed.
   e. Screw on bucket cap and keep buckets on ice.

⚠ CRITICAL: Corresponding rotor buckets should be balanced within 100 mg (total weight of rotor bucket, sucrose gradient and sample) of each other prior to ultracentrifugation to avoid machine errors.

Optional: Pre-chill ultracentrifuge to 4°C.

8. Ultracentrifuge gradients.
   a. For an SW-41 Ti rotor and Optima L-90K ultracentrifuge: centrifuge at 39,000 rpm (187,813 × *g*) for 1.5 h at 4°C.
   b. Set max acceleration and slow deceleration.
   c. Carefully transfer rotor buckets with sample from centrifuge and keep on ice.

⚠ CRITICAL: Slow deceleration is necessary to avoid disrupting polysome sedimentation.

⚠ CRITICAL: Keep centrifuge tubes in rotor buckets and store on ice until they are fractionated. Avoid disturbing the samples.

Note: We use the Brandel BR-188 Density Gradient Fractionation System integrated with Peak Chart software (v 2.08, Brandel, see http://www.brandel.com/fractgradient.html for more details). This system enables us to analyze the sample from top to bottom as sample is passed through an absorbance detector with 254 nm filters generating a continuous profile. We use a chase solution of 60% sucrose (w/v) containing bromophenol blue. A solution with a density greater than 50% sucrose is needed to push the sample from the bottom upward.

Bromophenol blue is used to distinguish chase solution from the sample. The Brandel system includes an integrated syringe pump that provides a constant flow rate. Fractionation occurred at a rate of 1.5 mL/min with each fraction containing 1 mL sample.

9. Using a density gradient fractionation system, fractionate and collect the samples into 1 mL fractions in 2-mL microcentrifuge tubes.
   a. Load syringe pump with chase solution and connect tubing.
      i. Connect syringe pump tubing to needle.
      ii. Remove all air bubbles in tubing by running syringe pump forward.
   b. Load the sample centrifuge tube into place.
      i. Carefully transfer sample tubes from centrifuge buckets and load into place.
      ii. Puncture bottom of tube with needle.
   c. Set syringe pump to 'computer control' and set rate to 1.5 mL/min.
   d. Click 'run' in the software and collect 11 sample fractions.
   e. Combine fractions into 4 pooled groups based on the absorbance profile: free, monosome, oligosome, and polysome, and label tubes. Downstream sample processing occurs on the set of combined fractions.

Note: We used PEAKS software (Brandel) to record polysome profiles at absorbance 254 nm.

Note: A small volume of fractions (approx. 200 μL) can be stored separately and used for downstream confirmation. Protein can be extracted from these samples and run-on western blot to confirm results of mass spectrometry.

Pause point: Ribosome fractions can be stored at −80°C.

Protein isolation and preparation for MS – Day 1

Timing: 48 h

In this step, proteins that have been separated into pooled fractions are isolated from the rest of the lysate/sucrose sample using TCA precipitation. Sample preparation for mass spectrometry includes reduction, alkylation, digestion, TMT-labeling, and fractionation of the isolated protein. TMT-labeling enables multiplexing of all sample fractions (DMSO and Silvestrol: ribosome-free, monosome, oligosome, polysome) into a single experimental run and facilitates peptide quantification.

10. Isolate total protein using TCA precipitation.
    a. To each 1 mL fraction add 250 μL TCA (20% final TCA concentration). Vortex and keep at 4°C for 1 h. Centrifuge at 10,000 × *g* for 10 min, 4°C.
    b. Aspirate supernatant.
    c. Wash protein precipitate with ice-cold acetone two times and aspirate.

⚠ CRITICAL: TCA is corrosive and should be handled with caution including the use of personal protective equipment. Additionally, acetone is flammable and toxic and should be used cautiously.

Note: We normally obtain roughly 10 mg of total protein (across all fractions) per sample.

11. Resuspend samples in 100 μL of reduction buffer and incubate at 65°C for 20 min.

Note: Allow samples to return to 22°C–25°C before proceeding to the next step.

12. To each sample, add 11 μL of 200 mM iodoacetamide (final concentration of 20 mM).

a. Incubate samples in the dark at 20°C–22°C for 20 min.

13. Add 450 μL acetone to each sample, vortex, and precipitate proteins at −20°C for 16 h.
    a. Centrifuge samples at 23,000 × *g* for 15 min.

⚠ CRITICAL: Acetone is flammable and toxic and should be used cautiously.

Pause point: Protein precipitates can be stored at −80°C.

14. Resuspend protein precipitates in 50 μL of NH_4HCO_3 (pH 8.3). Add 1 μL of MS grade Trypsin/LysC (final protease:protein ratio 1:50). Digest for 3 h at 37°C.
15. Dry samples using a vacuum concentrator. We use the Savant SPD2010 SpeedVac concentrator (more information can be found on the manufacturer's website: https://www.thermofisher.com/order/catalog/product/SPD2030-220?SID=srch-srp-SPD2030-220).
    a. Speedvac samples under 'manual run' and check on samples every half hour (may take 1–2 h).
16. Resuspend peptides in 0.1% TFA and desalt using standard C18/Ziptip cleanup.
17. Dry samples using a vacuum concentrator and perform TMT labeling. We use the TMT10plex Isobaric Label Reagent Set (Thermo Fisher) and follow the protocol according to the manufacturer's instruction: https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0016969_2162457_TMT10plex_UG.pdf.
    a. Combine labeled peptides from all samples and dry using a vacuum concentrator.
    b. Resuspend combined peptides in 20 μL ddH_2O.
18. Fractionate combined peptides using high pH reversed phase chromatography.
    a. Load samples on a C18 column (XBridge Peptide BEH C18 column, 300 Å, 5 μM, 4.6 mm × 250 mm) and elute with a 65 min gradient at a rate of 500 μL/min using Buffers A and B as following:
        i. 0–46 min, 4%-33 B.
        ii. 46–55 min, 70% B.
        iii. 55–55.1 min, 98% B.
        iv. 55.1–50 min, 98 B.
        v. 60–60.1 min, 3% B.
        vi. 60.1–65 min, 4% B.
    b. Begin fractionation after 2 min.
    c. Collect each fraction for 1.3 min (78 s) and collect a total of 44 fractions.
    d. Dry fractions with a vacuum concentrator.

Note: Fractionation begins after 2 min to avoid salt plug.

Pause point: Fractions can be stored at −20°C.

LC–MS/MS analysis– day 3+

Timing: 10–12 h

In this step, the combined and fractionated peptides are identified and quantified using LC–MS/MS. TMT-labeling enables peptide quantification and original sample identification (drug treatment, ribosome fraction).

Sample mass spectrometry

19. Resuspend sample peptide fractions in 0.1% formic acid, and load onto a 96-well plate for injection into the mass spectrometer (see note).
    a. Load peptide mixtures into trap column at a constant flow rate of 30 μL/min.

b. Elute peptides over the course of a 60 min gradient (see note).

Note: Instrument used in our study: Thermo Scientific Orbitrap Fusion-Lumos Tribid Mass Spectrometer (Thermo Fisher, San Jose, CA) outfitted with a nanospray source and Dionix Ultimate 1000 nano-LC system (Thermo Fisher, San Jose, CA).

Alternatives: This can work with any LC/MS setup and would work well any modern mass spectrometer such as the Orbitrap or the timsTOF series of instruments. If using other instruments, parameters simply need to be changed to tailor that instrument.

Note: Peptide mixtures were loaded into a PEPMAP100 C18 5 μM trap column (Thermo Fisher, San Jose, CA). Peptides were eluted and focused using a PEPMAP C18 2 μM 15 cm column (Thermo Fisher, San Jose, CA).

Note: 60 min gradient. 0–48 min :4%–35% acetonitrile + 0.1% formic acid; 48–55 min of 90% acetonitrile + 0.1% formic acid for column cleaning, 55–60 min of 4% acetonitrile + 0.1% formic acid for column equilibration. Peptides were introduced by nano-electrospray into the mass spectrometer.

20. Data acquisition.
    a. Acquire data using the MultiNotch MS3 acquisition with synchronous precursor selection (SPS) with a cycle time of 2 s.
    b. Perform MS1 acquisition (scan range of 550 m/z - 1,800 m/z with resolution set to 120 000, maximum injection time of 50 ms, and AGC target set to 4e5).
    c. Perform isolation for MS2 scans in the quadrupole, with an isolation window of 0.7 (conduct MS2 scans in the linear ion trap with a maximum injection time of 50 ms and a normalized collision energy of 35%).
    d. For MS3 scans, use higher-energy C-trap dissociation (HCD) with a collision energy of 65% and measure scans in the orbitrap with a resolution of 50 000, a scan range of 100 m/z - 300 m/z, an AGC Target of 1e5, and a maximum injection time of 50 ms. Dynamic exclusion was applied using an exclusion list of one repeat count with an exclusion duration of 30 s.

Sample mass spectrometry data analysis

21. Process MS raw files using PEAKS X+ (v10.5, Bioinformatics Solutions Inc.).

Alternatives: Any MS search software that allows for quantification should be able to search the dataset – including but not limited to PEAKS, MS Fragger,[5] MaxQuant,[6] Proteome Discoverer (Thermo Fisher), and Comet Search.[7]

Note: The data was searched against the Human Uniprot database consisting of reviewed canonical and isoform sequences (total entry 42339). Parent mass tolerance was set to 20 ppm, with fragment mass tolerance of 0.6 Da. Semi-specific tryptic cleavage was selected with allows for a maximum of 2 missed cleavages. Fixed modifications of TMT (229.162932 Da) on lysine and peptide N-terminal cysteine residues were specified. Carbidomethylation of cysteine (+57.02 Da) residues was selected as a fixed modification, while variable modifications included 13C6-15N2 SILAC on K (8.014199 Da), 13C6-15N4 SILAC on R (10.008269 Da), Oxidation of M (15.99 Da), and modifications of deamidation (0.98 Da) on asparagine and glutamine. TMT quantification was also performed using the PEAKS X+ quantification module, allowing a mass tolerance of 20 ppm and quantifying all peptides that pass a 1% FDR threshold.

Table 2. Abundance intensities for each ribosome fraction for most highly activated proteins

	Silvestrol				DMSO			
Gene name	Free	Mono	Oligo	Poly	Free	Mono	Oligo	Poly
CPSF3	37282	118290	28166	122220	78135	62085	0	21148
TP53BP1	1141800	136120	595400	105440	1366200	79571	239940	13551
SDCBP	35115	633360	81498	452760	93667	211470	143250	137430
NOL6	4079.3	43687	0	46644	28129	44683	0	46139
PROCR	27359	107100	47911	116270	35888	63558	0	24025

EXPECTED OUTCOMES

Ribosome density fractionation enables the separation of cellular protein synthesis machineries based on translational activity. Translation factors and RNA-binding proteins engaged in protein synthesis are enriched in the polysome fractions. Those involved in translation initiation are enriched in the ribosomal 40/60/80S fractions while those disengaged from translation are localized in the free fractions. SILAC is used to exclude the confounding presence of newly synthesized peptides and proteins by removing them from the analysis of active translation machinery.[8] An example of the ribosome fractionation profiles for vehicle and silvestrol are depicted in Figure 1B.We use as a primary readout the ratio of peptide/protein abundance in polysome fractions (active translation) to free fractions (translationally disengaged). A secondary readout can also be used taking the abundance ratio of the polysome fractions to the monosome (40/60/80S) fractions.[2,3] Silvestrol is compared to vehicle to determine factors actively engaged in translation during treatment. An example of protein quantitation output and an example of our primary and secondary readouts are depicted in Tables 2 and 3, respectively.

LIMITATIONS

In situations where treatment/compound causes near or complete inhibition of translation, the abundance of proteins in the polysome fractions may be below the limit of detection for mass spectrometers. This issue may be alleviated by either using more input material (cells) or by lowering the concentration of test compounds. Treatment for 24 h with 6.25 nM silvestrol leads to a 50% reduction in the area under the curve calculated for heavy polysome fractions compared to vehicle (Figure 1B, see Ho et al.[1]). Translational inhibition by silvestrol under these conditions manifests as a shift toward lighter ribosome density fractions.

Inter-operator variations may occur during the preparation of sucrose gradients. It is recommended that a single operator make all the gradients for each experiment. Alternatively, automated gradient makers are available commercially e.g., Biocomp gradient master.

TROUBLESHOOTING

Problem 1

Observe significant cell death / detachment during treatment, SILAC, or harvesting (steps 1–4).

Table 3. Abundance ratio of ribosome fractions used in primary and secondary readouts

	Primary readout Polysome / Free fractions			Secondary readout Polysome / Monosome fractions		
Gene name	Silvestrol Poly/Free	DMSO Poly/Free	Ratio Poly/Free	Silvestrol Poly/Mono	DMSO Poly/Mono	Ratio Poly/Mono
CPSF3	3.278	0.271	12.11	1.033	0.341	3.278
TP53BP1	0.092	0.010	9.310	0.775	0.170	0.092
SDCBP	12.89	1.467	8.788	0.715	0.650	12.89
NOL6	11.43	1.640	6.971	1.068	1.033	11.43
PROCR	4.250	0.669	6.348	1.086	0.378	4.250

Potential solution

The treatment conditions are likely too harsh for the specific cell line used. Rocaglate compounds are very potent and may require lower concentrations or shorter durations of treatment to obtain effect. Significant cell death confounds results and lowers yield RNA and protein needed for fractionation and downstream analysis. Some cell types may require additional care during steps 1–3 as small environmental changes may activate confounding stress responses.

Problem 2

Sucrose gradients that have not been properly prepared (step 2) will usually result in low-quality absorbance profiles (step 9) that lack clear delineation of the various components (free, monosome, oligosomes, polysomes).

Potential solution

A control tube can be included in steps 1 and 2, representing the group of tubes in that batch, to ensure sucrose gradients are accurately prepared. For the control tube, add a small amount of bromophenol blue to the 50% sucrose solution. Prepare the gradient as outlined: you should have a top clear layer and a blue bottom layer. Store horizontally at 4°C for 5–6 h. When the tube is raised vertically, you will see a linear gradient from clear (top, 10% sucrose) to dark blue (bottom, 50% sucrose). A gradient that has been disturbed or inaccurately prepared will result in the tube being all one color of blue, and these gradients will provide poor results. Alternatively, commercially-available gradient makers can be used and may limit operator variability.

Problem 3

Low absorbance 260 nm and poor resolution of peaks in polysome profiles during fractionation (step 9).

Potential solution

The 'estimated' RNA concentrations based on A260 in step 6 should be > 1,000 ng/μL. In our experience, this estimated concentration > 1,000 ng/μL reliably produces high resolution absorbance profiles and fractions containing enough material (RNA, protein) for downstream analysis. Ensure use of RNase inhibitor in lysis buffer in step 5. Dependent on the cell line being used, it may be necessary to increase number of cells in order to achieve adequate concentrations of RNA and protein.

Problem 4

Sample leaking during fractionation (step 9).

Potential solution

Wrap parafilm around the top of the ultracentrifuge tube to ensure sealed connections with other components.

Problem 5

How to confirm mass spectrometry results.

Potential solution

A small sample can be stored separately (see step 9 note) for confirmatory analysis. We typically use TCA precipitation to isolate protein, followed by western blotting to identify protein distribution and intensity across fractions. Alternatively, if sample is not saved, the experiment can be repeated, and protein isolated for western blot.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Stephen Lee (stephelee@med.miami.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Mass spectrometry proteomics data are deposited in the PRIDE repository and available via ProteomeXchange: PXD022556. https://www.ebi.ac.uk/pride/archive/projects/PXD022556.

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

J.J.D.H., J.H.S., and S.L. are responsible for the original conception design of the methodology and experiment. J.J.D.H., T.A.C., and J.K. carried out the experiments. J.J.D.H., T.A.C., J.K., S.L., and J.H.S. conducted analysis as detailed in the study by Ho et al.[1] T.A.C. wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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