Table 5: Troubleshooting PACT and PARS protocols.

Step	Problem	Potential reason	Solution
3, 5 (transcardial perfusion)	Incomplete exsanguination, or the absence of tissue stiffening with PFA perfusion	Catheter is not stably placed in the heart in order to deliver solutions into rodent vasculature; vasculature was compromised during initial hPBS flush because perfusion rate was too high; an insufficient amount of hPBS was pushed through vasculature such that blood remains in smaller vessels	Use a single suture (loop the thread around the aorta) or clip to secure the feeding needle in place at the level of the aortic arch; start the initial perfusion of hPBS at a slower rate, and flush twice the volume of hPBS through
6 (hydrogel monomer (HM) embedding)	Tissue damage during clearing; tissue seems to be unnecessarily fragile	Inadequate infusion of HM solution throughout tissue	It may be necessary to leave large tissue samples such as whole rat organs in HM for >12 h so that the monomer may fully penetrate the tissue
		Tissue is structurally fragile or delicate	Consider including PFA (1–4%) in HM formulation for subsequent sample preparations; extend the postfixation step
	Poor HM polymerization after 37 °C incubation	Inadequate degassing	Repeat degassing step (10 min under vacuum, 10 min of nitrogen bubbling) and 37 °C incubation
		Bad reagents	Use fresh PFA for fixation; prepare HM solutions immediately before use and store the thermoinitiator, acrylamide and bis-acrylamide stock solutions at 4 °C
	Embedded tissue or biological sample is too fragile for non-clearing applications (e.g., thin sectioning and imaging)	Insufficient density of tissue cross-linking	Increase the concentration of PFA (1–4%) and/or include bisacrylamide (0.05%) in the HM formulation
7 (tissue clearing)	Clearing rate appears to slow down before the tissue is clear	Clearing may slow down as the clearing buffer acidifies	Buffer-exchange the clearing solution
		Dense cross-linking	If A4P1–4 was used, remove PFA from PACT hydrogel formulation in subsequent experiments; reduce the PFA postfixation incubation time by half
		Tissue is dense, highly myelinated and/or otherwise difficult to clear	Continue incubating in clearing buffer while checking periodically. Consider PARS clearing rather than PACT clearing for peripheral organ samples, as perfusive force accelerates clearing rate
	Tissue appears to degrade	Bacterial contamination	Buffer-exchange the clearing solution, adding 0.01–0.05% (wt/vol) sodium azide to PBS-based clearing solutions
		Poor hybridization of tissue to HMs	In subsequent clearing experiments, prepare the hydrogel monomer solution with fresh reagents, increase the PFA content by 1%, extend the tissue incubation in HM by 12–24 h and/or before polymerizing the tissue-hydrogel, perform two rounds of degassing (where one round equals 10 min under vacuum and 10 min nitrogen-bubbling)
		Poor PFA cross-linking of tissues	Ensure that adequate fixation and postfixation steps are performed; use fresh 4% PFA
	Hydrogel softening during clearing	Overclearing and/or initial poor hydrogel polymerization	Consider doubling the postfixation step or including PFA in the HM formulation in subsequent experiments; consider underclearing tissue, as RIMS incubation will cause translucent tissues to become transparent for imaging
	Difficulty obtaining complete bone decalcification	PACT-deCAL procedure requires further optimization by the user according to the bone size and density (guidelines provided are specific to the mouse femur and tibia)	Experiment with EGTA-based chelation and then 8% (wt/vol) SDS clearing. Alternate steps for '7B PACT-deCAL' are as follows: (i) Incubate bone-hydrogel in 0.1 M EGTA in 1× PBS (pH 9) for 72 h at 37 °C. (ii) Rinse the sample in 1× PBS; clear it in 8% SDS-PBS (pH 7.4) for 7 d at 37 °C, performing one buffer exchange during clearing. (iii) Wash the sample as usual: 24–48 h in 3–6 buffer changes of 1× PBS at RT

Step	Problem	Potential reason	Solution
		Dense, fibrous bone or larger samples may be resistant to decalcification by chelating reagents and SDS-based clearing alone	As bone consists of ~16% collagen[196, 197], consider incubating bone in collagenase before clearing in order to disrupt the collagen matrix
	Tissue becomes turbid; white precipitate appears in the tissue	Incomplete washing after clearing, causing SDS and/or salts to precipitate in tissue when it is moved from 37 °C to RT	Double the time for of all wash steps, making sure to perform several exchanges of 1× PBS each day; wash with PBST or BBT instead of 1× PBS
		Tissue becomes white and nearly opaque upon transfer to 4 °C	Salts and, in particular, residual SDS will precipitate in tissue if it is moved to 4 °C. However, the precipitate should disappear upon gradual warming of tissue to RT or 37 °C. Consider performing more extensive wash steps in future experiments, particularly after SDS clearing
	Slight tissue yellowing during clearing	Use of PFA-containing hydrogels or BB	We have not observed adverse effects from slight tissue yellowing on imaging results—tissue becomes clear upon RIMS mounting. However, very occasionally, some samples become very yellow during the first half of SDS clearing: these samples should be cleared for a longer length of time—until they are very transparent—or the yellowing will cause high background during imaging. Ensure that only fresh PFA is used in subsequent experiments
	Brain does not become transparent during PARS-based clearing	Insufficient perfusion with clearing buffer	Extend the clearing time: most rodent organs clear within 2 d via PARS; however, the brain requires an additional 1–2 weeks to clear. RIMS-mounting will also increase the transparency of 'translucent' tissues
	A specific organ does not clear well via whole-body PARS	Vasculature becomes compromised during the clearing process	Identify and try to fix leakages in the vasculature; if unsuccessful, tie off the major vessels supplying that organ, excise the organ for PACT clearing and continue to perform PARS clearing with the remaining body. Starting over with a new PARS preparation should only be used as the last resort
		Poor flow to specific organ because of anatomic reasons (poorly vascularized)	If PACT is not a desirable option and the organ is sizable with accessible vasculature, consider PARS clearing the single organ, akin to published decellularization methods[92]
9 (histology)	Poor labeling, including faint signal	Shallow antibody penetration	Increase the antibody concentration in the primary antibody cocktail or replenish the antibody halfway through extended incubations, by either adding additional antibody directly to the original antibody cocktail or by preparing a fresh antibody dilution
		Incomplete delipidation, which obstructs labeling	Increase the clearing time
		High cross-linking density	High cross-link density in A4P1-4–hybridized tissues will slow antibody diffusion; thus, antibody incubations should be extended
		Epitope loss or epitope masking (unlikely if adhering to protocol)	If tissue was damaged because of microbial contamination, consider adding 0.01–0.05% (wt/vol) sodium azide to all buffers and solutions that are used in long incubations; overfixation may lead to antigen masking, so postfixation steps should be decreased
		In FISH experiments, degradation of nucleic acid transcripts, or diffusion of transcripts out of sample during clearing	Ensure that all hydrogel, clearing and labeling reagents are RNase-free; embed samples in a hydrogel formulation that contains PFA and/or bis-acrylamide (e.g., A4P1B0.05), and perform a rigorous degassing step to ensure thorough hydrogel-tissue hydridization
		Poor quality of antibody or dye, which results in weak labeling	Only use high-quality antibodies that have been first verified in standard thin-section immunolabeling; experiment with a different antibody supplier—different antibodies against the same target may vary greatly in their labeling abilities, such as in their binding affinity and in their capacity to access intracellular compartments for cell-filling labeling versus only superficial or extracellular epitope binding. Finally, it can be helpful to simultaneously prepare a thin section (40–100 μm) alongside a thick, cleared section while troubleshooting to ensure that the visualization of a strong signal is possible

Step	Problem	Potential reason	Solution
	High background and/or autofluorescence	Tissue damage during processing	Review procedures carefully, and ensure that no reagents introduced bacterial contamination of sample; lengthen the wash steps to remove potential precipitate (SDS, donkey serum–antibody immunocomplexes)
		Sources of autofluorescence—part 1: fixative-induced autofluorescence, elastin, collagen	Many standard histological techniques for reducing autofluorescence, such as tissue bleaching[23], performing wash steps in PBST containing 100 mM glycine to quench aldehydes and treating tissue with histology stains that quench or mask autofluorescence, may be adapted to thick-sectioned cleared tissues—typically by performing longer wash steps after the appropriate countermeasure; photobleaching the tissue before IHC at wavelengths that exhibit the highest autofluorescence may also help[198]
		Sources of autofluorescence—part 2: heme chromophores, lipofuscins	Thoroughly remove all blood during initial cardiac perfusion; to elute heme, incubate hydrogel-embedded PACT sections and in particular PACT-deCAL sections in aminoalcohol (CUBIC reagent-1 (refs. 11,21) for 12–24 h at 37 °C with shaking, and then transfer the sections directly into 8% (wt/vol) SDS for clearing; lipofuscin autofluorescence is partially combatted by tissue clearing; however, thick tissue sections may be incubated in 0.2%[199] to 1.0% ((wt/vol) final concentration) Sudan Black B for 1–3 hours immediately before Step 5 (PACT hydrogel-embedding) in order to reduce high autofluorescent background—tissue clearing will allow Sudan Black B–treated sections to become sufficiently transparent for imaging (Supplementary Fig. 3)
	High background, but with high signal of correctly labeled epitopes	Nonspecific antibody binding	Extend the wash steps after both primary and secondary antibody incubations an additional day, by performing four or five buffer exchanges each day, and wash the samples in PBST instead of 1× PBS; in rodent tissue samples, avoid using antibodies that require anti-mouse secondary antibody labeling[23]; also some chicken antibodies show strong staining with high background and/or aggregation—these antibodies should be diluted to 1:400 to 1:1,000
12, 13 (tissue mounting and imaging)	Poor image quality and/or poor imaging depth	Tissue is of insufficient transparency for light to penetrate	Extend the tissue incubation time in RIMS to several days before imaging; for bone, incubate for an additional 1 d in RIMS-1.48 or RIMS-1.49 before imaging
	Morphological distortion	Tissue size fluctuations	Immediately before RIMS incubation, postfix cleared, immunolabeled tissue in 4% PFA for a few hours at RT, and then wash and incubate in RIMS for at least several days to one week before imaging; consider preparing future samples in hydrogel that contains PFA (e.g., A4P1–4, depending on the degree of swelling) and/or consider a longer postfixation step after transcardial perfusion
	Bubbles in mounted tissue	Air trapped in tissue or dissolved air in RIMS; sample mounted with insufficient RIMS, causing the introduction of air bubbles between the RIMS meniscus and cover glass	Purge RIMS of excess air via degassing the tissue in fresh RIMS before mounting (e.g., using the vacuum line, akin to the hydrogel polymerization of Step 5; do not bubble nitrogen through the sample following its placement under vacuum)—use this degassed RIMS to mount the degassed sample
	Sample appears turbid or white	RIMS-mounted sample was placed at 4 °C, causing salts, etc., to precipitate	The precipitate should disappear upon gradual warming of tissue to RT or 37 °C. Store RIMS-mounted tissue at RT, protected from light, or mount tissue in cRIMS for cold storage
16, 17 (3D image analysis)	Imaging software and/or computer crashes; unable to load acquired images	Insufficient RAM for large images	Troubleshoot with a different option in the step 15 workflow: option A using Imaris, option B using TerraStitcher, or option C Vaa3D TerraFly; consider upgrading computer workstation and/or adding RAM and/or new graphics card; downsample the data set (note that compression cannot be used with Imaris); process the images in tiles (i.e., analyze each tile individually)