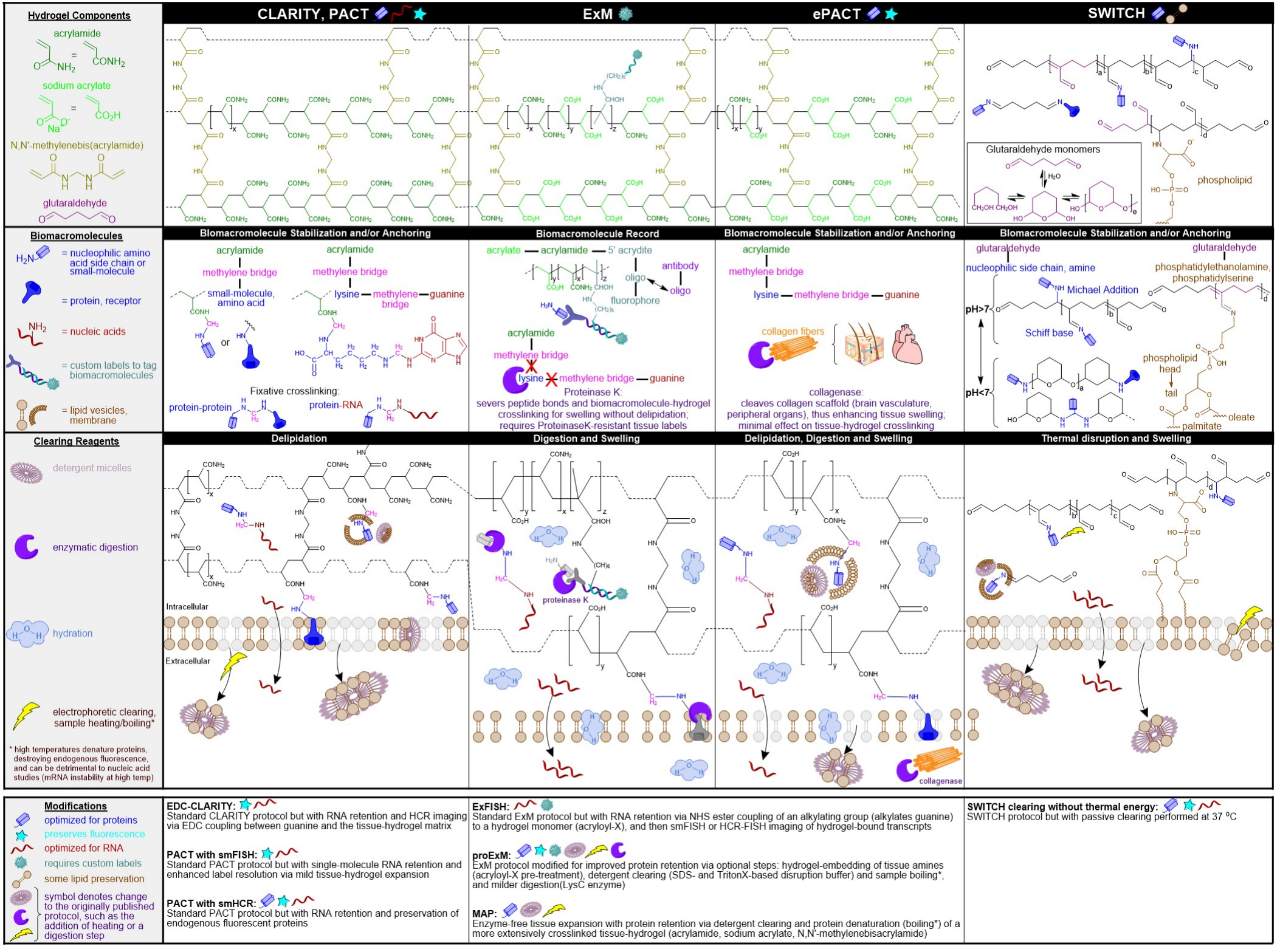
Supplemental Material: Annu. Rev. Biophys. 2018. 47:355–76
https://doi.org/10.1146/annurev-biophys-070317-032905
Hydrogel-Tissue Chemistry: Principles and Applications
Gradinaru et al.

Supplemental Figure 1. Integration of native biomolecules as HTC gel constituents.

Expanded form of Figure 4. Hydrogel-tissue chemistry (HTC) backbone variants (selected subset shown, *top row*) allow selective and customizable biological macromolecule anchoring and functionalization (*middle row*). Molecular architecture and macromolecule-anchoring properties of the initial CLARITY formulation (top left; 16) and some subsequent variants are shown. The chemical backbone of the CLARITY hydrogel interacts with tissue elements through two principal routes: (*i*) covalent conjugation to amine-containing and otherwise functionalized proteins, nucleic acids, and small biomolecules; and (*ii*) noncovalent trapping of bulky moieties, such as extensively cross-linked protein networks within the hydrogel matrix. Exhibiting these similar interactions with tissue components, the subsequent HTC variants are also capable of preserving molecular information during tissue processing through physically securing tissue macromolecules to the hydrogel or through recording their cellular location using custom labels that can withstand processing steps. All of these approaches incorporate native biomolecules as an integral and essential part of the tissue-hydrogel matrix, including via (*i*) targeted retention of specific biological macromolecules [nucleic acids with EDC-CLARITY (123), PACT (140)/smFISH/smHCR and ExFISH (12); and lipids with SWITCH (92)]; (*ii*) increased rigidity (SWITCH); or conversely (*iii*) increased size-flexibility [clearing reagents and modifications: PACT (140)/ExM (11)/ePACT (131)/proExM (125)/ExFISH (12) shown, iExM (10) and MAP (62) not shown]. (*bottom row*) To visualize the macromolecular composition of whole tissue samples, this molecular information was originally accessed via detergent-based delipidation, with or without electrophoretic acceleration (16). Many subsequent methods (PACT (140), ePACT (131), EDC-CLARITY (123), and PACT with smFISH or smHCR) retained this use of sodium dodecyl sulfate (SDS) for clearing samples at moderate temperatures and at neutral or slightly alkaline pH due to the important compatibility of these mild conditions for maintenance of endogenous fluorescence and of nucleic acid and protein content. Other methods (SWITCH, MAP) (62, 92) found distinct benefits in varying temperature and pH, alterations that can accelerate lipid removal and improve size flexibility of the tissue-hydrogel matrix at the cost of losing some molecular content. ExM (11) employs enzymatic digestion of all tissue proteins and hydration; although biomolecular information and endogenous fluorescence are compromised, individual labeling with customized probes forms a record of their original presence. Of subsequent modifications [ExM with antibodies, ExFISH (12), iExM (10), and proExM (125)],

proExM converges back toward resembling the CLARITY/PACT/ePACT protocols, with only mild enzymatic digestion or heated incubation in an alkaline SDS solution en route to expansion/clearing. The custom trifunctional linker/label depicted in the original ExM protocol (11) consisted of three conjoined components: 1. a biomacromolecule recognition moiety, such as an oligonucleotide capable of hybridizing with a complementary sequence that has already been appended to an antibody, 2. a fluorescent tag, and 3. a monomer-like methacryloyl functional group covalently incorporated into the hydrogel network during polymerization. In proExM (125) and variation ExPath (146), these three linker components are replaced with a single small-molecule linker for biomacromolecule recognition and hydrogel incorporation; succinimidyl ester of 6-((acryloyl)amino)hexanoic acid (AcX) for modifying amines, including fluorescently-tagged secondary antibodies, with a polymerizable acrylamide functional group. In ExFISH (12), a bifunctional linker is formed from two components: the AcX small-molecule of ProExM, and a second small-molecule containing both an amino group, which mediates the attachment to AcX, and an alkylating group, which serves as the biomacromolecule recognition moiety via its reaction with the N7 of guanine in RNA. Finally, iterative ExM (iExM (10) uses two (or more) composite polyelectrolyte gels and two AcX-based linker types; the first is an oligonucleotide conjugated to a polymerizable acrydite moiety, while the second is a complementary oligonucleotide with a second acrydite moiety and a fluorescent tag at either end. Like ExM, the first oligonucleotide linker is designed to hybridize to a complementary sequence appended to an antibody. Abbreviations: EDC, 1-Ethyl- 3-3-dimethyl-aminopropyl carbodiimide; ePACT, expansion passive CLARITY technique; ETC, electrophoretic tissue clearing; ExFISH, expansion fluorescent in situ hybridization; ExM, expansion microscopy; iExM, iterative expansion microscopy; MAP, magnified analysis of the proteome; PACT, passive CLARITY technique; proExM, protein-retention expansion microscopy; SWITCH, system-wide control of interaction time and kinetics of chemicals; smFISH, single-molecule fluorescent in situ hybridization; smHCR, single-molecule hairpin chain reaction.

(See figure on following page)


Hydrogel Components
acrylamide
sodium acrylate
N,N'-methylenebis(acrylamide)
glutaraldehyde
CLARITY, PACT
ExM
ePACT
SWITCH
Glutaraldehyde monomers
phospholipid
Biomacromolecules
= nucleophilic amino acid side chain or small-molecule
= protein, receptor
= nucleic acids
= custom labels to tag biomacromolecules
= lipid vesicles, membrane
Biomacromolecule Stabilization and/or Anchoring
acrylamide
methylene bridge
small-molecule, amino acid
lysine
methylene bridge
guanine
or
Fixative crosslinking:
protein-protein
protein-RNA
Biomacromolecule Record
acrylate
acrylamide
5' acrydite
antibody
oligo
fluorophore
Proteinase K:
severs peptide bonds and biomacromolecule-hydrogel crosslinking for swelling without delipidation;
requires ProteinaseK-resistant tissue labels
Biomacromolecule Stabilization and/or Anchoring
collagen fibers
collagenase:
cleaves collagen scaffold (brain vasculature, peripheral organs), thus enhancing tissue swelling;
minimal effect on tissue-hydrogel crosslinking
Biomacromolecule Stabilization and/or Anchoring
glutaraldehyde
nucleophilic side chain, amine
Michael Addition
Schiff base
pH>7
pH<7
phosphatidylethanolamine, phosphatidylserine
phospholipid head
tail
oleate
palmitate
Clearing Reagents
detergent micelles
enzymatic digestion
hydration
electrophoretic clearing, sample heating/boiling*
* high temperatures denature proteins, destroying endogenous fluorescence, and can be detrimental to nucleic acid studies (mRNA instability at high temp)
Delipidation
Intracellular
Extracellular
Digestion and Swelling
proteinase K
Delipidation, Digestion and Swelling
collagenase
Thermal disruption and Swelling
Modifications
optimized for proteins
preserves fluorescence
optimized for RNA
requires custom labels
some lipid preservation
symbol denotes change to the originally published protocol, such as the addition of heating or a digestion step
EDC-CLARITY:
Standard CLARITY protocol but with RNA retention and HCR imaging via EDC coupling between guanine and the tissue-hydrogel matrix
PACT with smFISH:
Standard PACT protocol but with single-molecule RNA retention and enhanced label resolution via mild tissue-hydrogel expansion
PACT with smHCR:
Standard PACT protocol but with RNA retention and preservation of endogenous fluorescent proteins
ExFISH:
Standard ExM protocol but with RNA retention via NHS ester coupling of an alkylating group (alkylates guanine) to a hydrogel monomer (acryloyl-X), and then smFISH or HCR-FISH imaging of hydrogel-bound transcripts
proExM:
ExM protocol modified for improved protein retention via optional steps: hydrogel-embedding of tissue amines (acryloyl-X pre-treatment), detergent clearing (SDS- and TritonX-based disruption buffer) and sample boiling*, and milder digestion(LysC enzyme)
MAP:
Enzyme-free tissue expansion with protein retention via detergent clearing and protein denaturation (boiling*) of a more extensively crosslinked tissue-hydrogel (acrylamide, sodium acrylate, N,N'-methylenebisacrylamide)
SWITCH clearing without thermal energy:
SWITCH protocol but with passive clearing performed at 37 °C