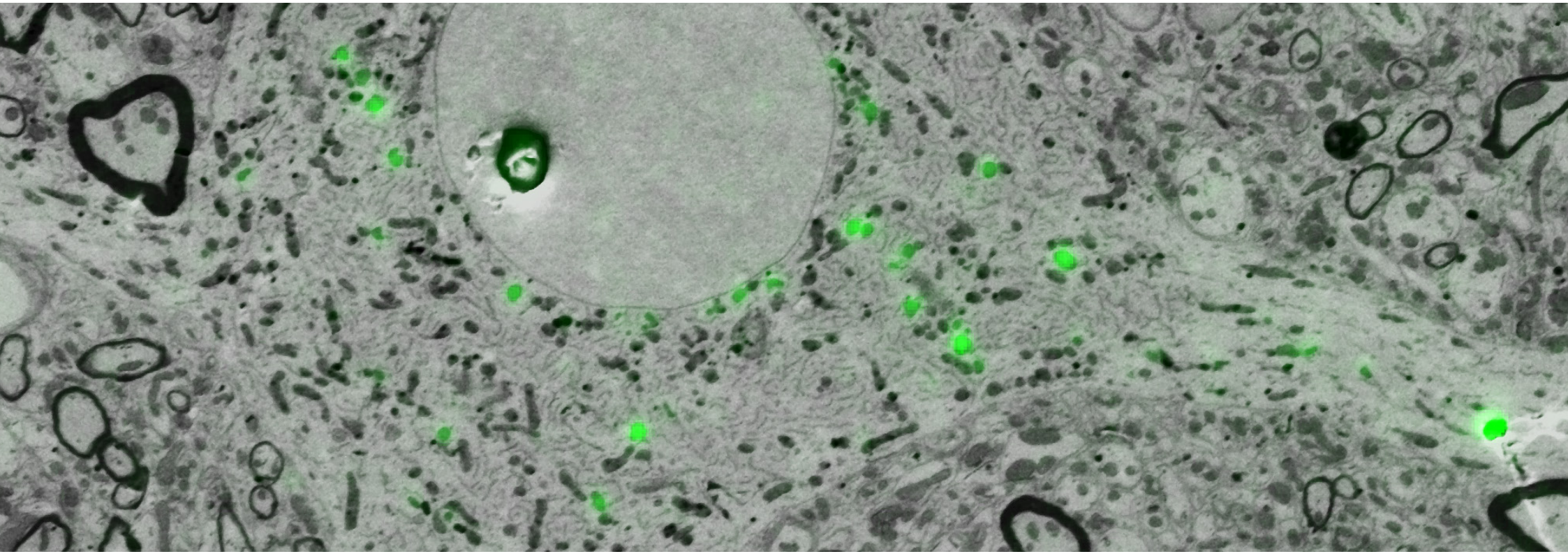

SAMPLE PREPARATION

for Integrated Correlative Light and Electron Microscopy

delmic

Integration without compromise

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Sample Preparation for Integrated Correlative Light and Electron Microscopy

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Integrated correlative light and electron microscopy (CLEM) offers the possibility to study the same area on a sample using both fluorescence microscopy and electron microscopy. One of the challenges associated with integrated CLEM (iCLEM) is the preparation of samples suitable for both FM and EM. Here, we discuss several methods suitable for an integrated imaging workflow, and present results obtained using a variety of techniques and samples, including: cultured cells and tissue sections, chemical fixation or cryo-fixation, genetic labels and immunolabeled samples. While particularly suited for iCLEM, these protocols may also improve correlation in non-integrated approaches or a combination of both. This is not intended as an extensive list of sample preparation methods, which are many and varied. Furthermore, different types of specimens typically require specifically optimized protocols. This short review provides insights in the possibilities offered by integrated imaging workflows and represents a useful starting point for exploring these techniques.

INTRODUCTION

During the past few years, correlative light and electron microscopy (CLEM) has gained in popularity as a research tool. This growing interest is because CLEM combines the strengths of fluorescence microscopy (FM) and electron microscopy (EM): FM is the ideal tool to collect functional information about specific components inside tissues, cells and organelles; EM offers substantially higher resolution and can provide detailed contextual structural information. FM can thus be used to pinpoint regions of interest for subsequent higher resolution EM.

Until recently, however, CLEM has been challenging, costly, time consuming and thus requiring high levels of expertise. Correlative methods normally require two distinctly different imaging setups which are traditionally located in separate facilities, and the sample preparation methods for each tend to be incompatible. Due to the fundamental differences between microscopes, extra sample preparation steps are also usually necessary when switching from FM to EM. This often distorts the sample, hampering accurate correlation. In addition, it can be extremely challenging to relocate a region of interest originally identified with FM in EM since the information used to navigate in FM is not visible in EM, and this problem becomes more significant as the size of specimen increases.

Integrated CLEM (iCLEM) overcomes most of these difficulties. By integrating fluorescence and scanning electron microscopy, the need to transfer between two different microscopes is eliminated. Finding back a region of interest becomes much simpler as the same area of the sample is observable with both microscopes. Furthermore, since the sample is not subjected to intermediate preparation steps, its conformation is guaranteed to be identical.

Sample preparation for iCLEM is also a new research area, and a limited number of protocols have been published to date. For non-integrated CLEM, on the other hand, excellent overviews of sample preparation methods exist [1-3]. One of the difficulties of integrating sample preparation for FM and EM, is that EM sample preparation protocols typically use heavy metal stains to introduce electron contrast. It is well known that these heavy metals can quench nearby fluorescence. Furthermore, EM requires vacuum compatible samples. As such, samples need to be dried, which can influence the amount of fluorescence for hydration-sensitive dyes [4]. A recent publication has also shown that in SEM the amount of fluorescence can be influenced by variation of the vacuum pressure [5]. As iCLEM develops further as a powerful research tool, we will see a corresponding increase in the number of published iCLEM specific sample preparation protocols.

Here we present four different protocols for iCLEM. We have deliberately chosen to use examples with

different types of samples, and varied preparation techniques. Another consideration was to focus on (where possible) relatively simple protocols, thereby allowing many alterations and variations to be made.

Please note that this document is not meant to be an extensive overview of sample preparation protocols for iCLEM. Furthermore, we will not discuss the biological relevance or the application for which each protocol was developed. Rather, this document is intended as an introduction into the possibilities of sample preparation for iCLEM. Our goal is to demonstrate that there are no fundamental limitations for integrated sample preparation. We also show that iCLEM can be extremely fast, and crucially, that it is possible to generate very accurate overlays with no additional image manipulation.

METHODS & RESULTS

The SECOM platform (DELMIC B.V.) was used for correlative imaging. The sample mounting procedure is therefore specifically designed for this platform, and is illustrated in Fig. 1.

Samples are placed (or grown) directly on cover glasses coated with indium tin oxide (ITO). A thin coating of ITO is transparent to visible light, as well as conductive, and allows imaging of uncoated biological samples in a scanning electron microscope (SEM) [6].

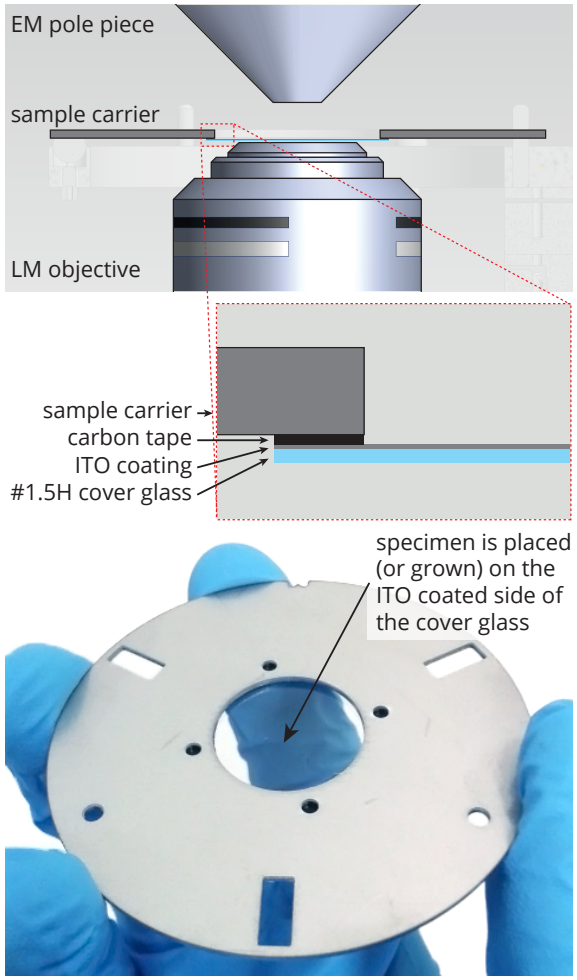


Fig. 1. *Illustration of how the specimen is placed inside the SECOM platform. The e-beam scans from above, whereas the light microscope objective is situated below the cover glass. To prevent charging of the sample due to the e-beam, the cover glass is coated with indium tin oxide (ITO) and connected to the sample carrier using carbon tape.*

The results from each protocol are presented in Fig. 2. Short summaries of each method can be found at the end of this document, and we refer you to their respective cited publications for a complete description of the protocol.

Songbird brain

Understanding synaptic connectivity is essential to extending our knowledge of neural mechanisms. The combination of EM, capable of resolving synaptic vesicles and post-synaptic densities, and fluorescent markers allows synapses observed in the EM to be associated with specific neuron types [7].

It is interesting to note that even though the EM staining used in this study quenched the initial fluorescence of the tracers, the tracer was able to be re-labeled after sectioning using fluorescent antibodies, demonstrating that the protocol preserved antigenicity well enough to allow for on-section immunolabelling. Though this protocol is specific to neurological samples, it is a very interesting application and could be adapted for extension to other applications.

HeLa cells

In this study, the goal was to investigate the distribution of the lipid diacylglycerol within cellular membranes [5]. To do this, a protocol was developed that preserves GFP and mCherry fluorescence whilst retaining electron contrast in resin-embedded sections. For a full description and details of the different embedding media that were tested, please refer to the original research article [5]. One of the interesting findings is that the authors argue that the use of a quick freeze substitution protocol [8] might be essential to preserve the fluorescence of GFP for iCLEM.

Zebrafish

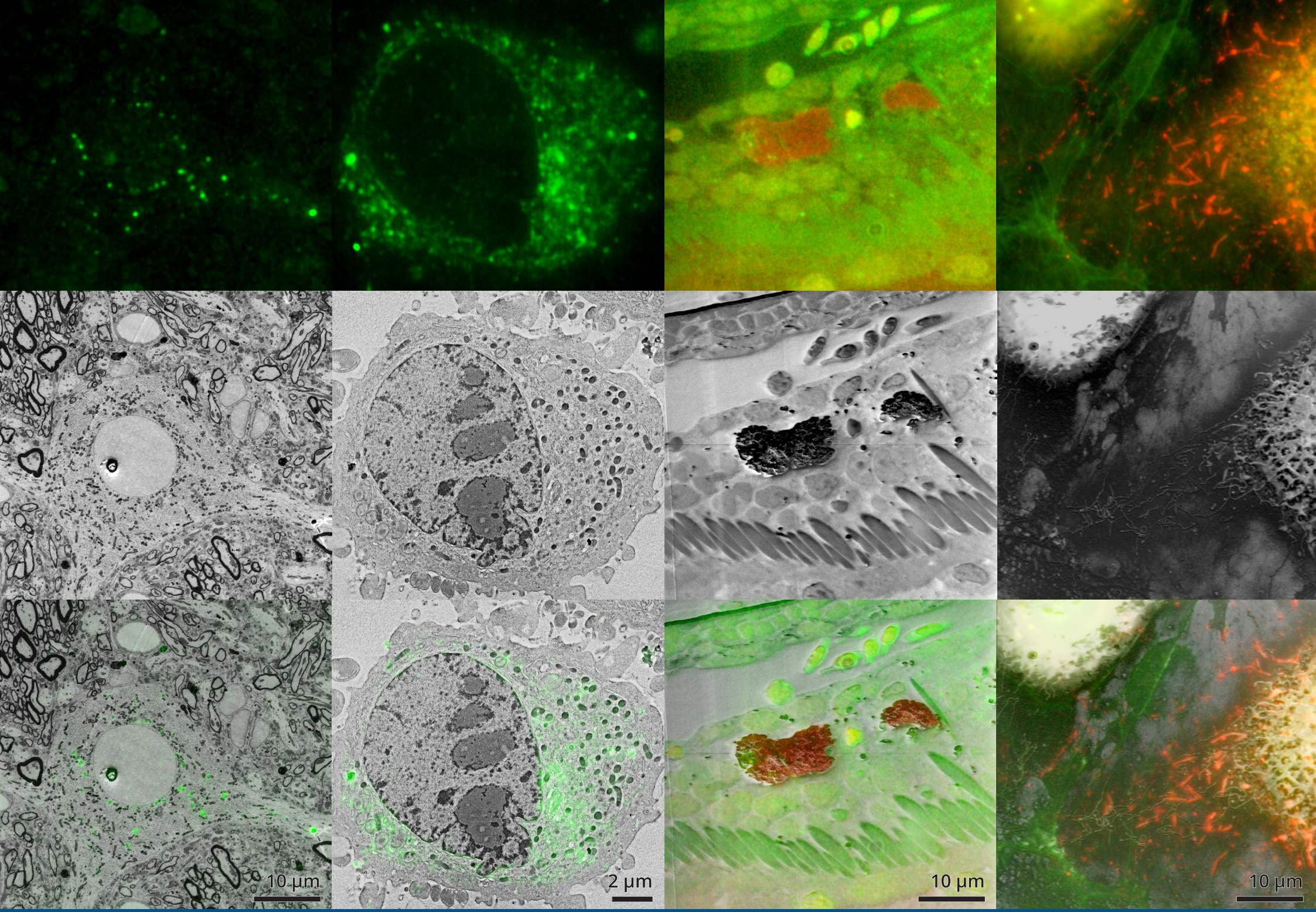
Because heavy metal staining clearly influences fluorescence, we decided to experiment with sample preparation protocols without any heavy metal staining. In this way, the fluorescence signal is optimally preserved. The adverse effect is that the level of electron contrast is significantly reduced. Nevertheless, it is clear that there is still a considerable amount of contrast in EM mode. The cell membranes, however, are not visible. Follow up experiments are currently being performed with low amounts of osmium tetroxide.

In FM mode, the GFP and E2-Crimson signals were easily detectable. It must be noted that the red structures in Fig. 2 are actually pigment cells and not due to specific labelling. The mCherry signal was present in other parts of the embryo (data not shown).

HUVEC

Human umbilical vein endothelial cells (HUVEC) contain rod-like storage granules called Weibel-Palade bodies which contain Von Willebrand factor (VWF). These organelles play an important role in blood coagulation. Here, the goal was to image these rod-like structures in the thin parts of the cell where they can be seen under the cell membrane using the SEM.

Fig. 2. *Correlative light and electron micrographs using the SECOM platform (DELMIC B.V., Delft) installed on a Quanta 250 FEG (FEI Company, Eindhoven). 1st row: fluorescence image. 2nd row: scanning electron micrograph. 3rd row: overlay of FM and EM. Columns 1 to 4: projection neurons in songbird brain, HeLa cell expressing GFP-C1, Zebrafish and human umbilical vein endothelial cells labeled for Von Willebrand factor. Columns 1, 3 and 4: EM imaging using seconday electron detector and FM imaging with Nikon Plan Apo 60x /0.95 lens, multicolor LED light engine, Clara CCD camera (Andor Technology, Belfast). Column 2: EM imaging using the vCD backscatter detector and FM imaging with Nikon Plan Apo 100x /1.40 oil immersion lens using vacuum compatible immersion oil, laser light source, Zyla sCMOS camera (Andor Technology, Belfast).*


10 µm
2 µm
10 µm
10 µm

We used a very fast sample preparation protocol where fixation, immunolabeling, dehydration and correlative imaging were performed in one day. Since whole cells typically display good enough contrast when imaged at low accelerating voltages, no additional EM staining was used [9].

The fluorescent signal was preserved remarkably well, and after storing the dried sample in a refrigerator for a month, the samples still displayed enough fluorescent signal for imaging.

OUTLOOK

In this preliminary review, we have illustrated different sample preparation possibilities for iCLEM, each of which uses a different approach on a variety of samples. These methods demonstrate that it is possible to find an integrated sample preparation solution for a wide range of applications. The neurology application example provides a good demonstration for on-section immunolabelling of resin embedded material, whilst the HeLa cell example shows that it is possible to retain GFP fluorescence in resin using freeze-substitution.

It is clear that the protocol used for Zebrafish is very much a work in progress with many opportunities for improvement. Nevertheless, we included this protocol together with that for HUVECS since each shows that even without additional contrast enhancement, the level of detail available using EM is sufficient. Furthermore, these protocols demonstrate the potential of straightforward sample preparation methods for iCLEM. Depending on the specific research question of interest, these protocols can be modified or extended to deliver valuable results.

Although fluorescence preservation and intensity is clearly influenced by the restraints of an integrated approach, we have shown that a suitable balance between EM and FM contrast can be found. Where this balance lies and how it can be achieved will obviously vary for each experiment, and as such, it is advisable to optimize sample preparation protocols for each application.

The main advantages of integrated preparation methods are the absence of intermediate specimen preparation steps when moving from FM to EM, and the high degree of correlation accuracy with minimal or no image manipulation over both small and large fields of view. As such, integrated solutions offer a streamlined imaging workflow which is both faster and more accurate.

ACKNOWLEDGEMENTS

Songbird brain samples were kindly provided by Dr Thomas Templier and Prof Dr Richard H R Hahnloser, University of Zurich and ETH Zurich. Sample preparation and imaging of transfected HeLa cells was performed by Dr Christopher J Peddie and Dr Lucy M Collinson, Cancer Research UK London Research Institute. Zebrafish samples were provided by Rohola Hosseini and Gerda Lamers, Leiden University. We gratefully acknowledge the help of Marjon J. Mourik, Leiden University Medical Center, with the sample preparation of HUVECs.

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Protocol: Songbird brain [1]

Surgery

Anesthetize		isofluorane (2% in O2)
Inject		~0.5 µl different conjugated dextrans
Inject tracers		Dextran Alexa 488 and Dextran Texas Red
Lethal dose		pentobarbital

Fixation

Perfuse		20 µl heparin and 5 ml 0.9% NaCl
Fix	20 min	2% PFA and 0.075% GA in phosphate buffer (0.1 M, pH 7.4)
Remove brain		
Post-fix	1 h	2% PFA and 0.075% GA in phosphate buffer (0.1 M, pH 7.4)

Cut 60-µm-thick sagittal vibratome sections
Localize the area of interest using a widefield fluorescence microscope

Electron microscopy staining

Wash		cacodylate buffer (0.1 M, pH 7.4)
post-fix	40 min	1.5% potassium ferrocyanide and 1% OsO_4 in cacodylate buffer (0.1 M, pH 7.4)
	1 h	1% OsO_4 in cacodylate buffer (0.1 M, pH 7.4)
	1 h	1% uranyl acetate in distilled water
Dehydrate		
Flat-embed		Durcupan ACM resin, cure for 48 h at 52°C

Sectioning

Localize the area of interest using a light microscope
Resection and attach to a blank resin block
Serial section, 60–90 nm
Collect sections on ITO coated coverslips

Immunofluorescence staining

Etch	10 min	1% periodic acid
Wash 15 times		ddH_2O
Wash 2 times	10 min	Tris and PBS (TPBS, pH 7.4)
Pre-block	30 min	5% goat serum in TPBS
Block	10 min	1% goat serum in TPBS
Primary	1.5 h	1:50 Rabbit anti Alexa 488 and 1:50 Rabbit anti Texas Red
Wash 4 times	10 min	Tris–HCl buffer (0.05 M, pH 7.5)
Secondary	1.5 h	1:50 Alexa 546 anti-rabbit
Wash 15 times		ddH_2O

Protocol: Transfected HeLa cells [2]

Cell culture

Maintain cells in DMEM supplemented with 10% foetal bovine serum
Transfect cells with GFP-C1 and mCherry-H2B constructs
Fixation was performed 18–24 h after transfection

High pressure freezing

Spin cells in an Eppendorf tube to form a pellet
Resuspend in equal volume of media and 10% BSA, maintain at 37°C
Spin down a volume of cells in a blocked 200 µl pipette
Cut away the end of the tip and pipet into membrane carriers
Load into high pressure freezer
Store carriers containing frozen cells under liquid nitrogen

Quick freeze substitution

Modified version of the method described in [8]

Substitution media		5% H_2O and 0.1% or 0.2% UA in in acetone

Transfer to moulds filled with 100% acetone and incubate for 15 min

Wash 3 times	15 min	100% acetone
Infiltration	3h	20%, 40%, 60%, 80%, 100% HM20
Incubate	overnight	100% HM20
4 changes of fresh resin		
polymerize	48h	360 nm UV light

Sectioning
Trim blocks from the moulds and store at room temperature in the dark

Trim away membrane carriers by hand
Cut and trim perpendicular to the cell layer
Serial section, 200 nm
Collect sections on ITO coated coverslips

Protocol: Zebrafish

Labels
GFP and E2-Crimson

Fixation

Fixation	2 h	PHEM fixative
Rinse 3 times	10 min	PHEM

Dehydration
Dehydration and resin Infiltration on a rotator

Dehydrate		15 min 50%, 15 min 70%, 15 min 80%, 15 min 90%, 15 min 100% Ethanol

Embedding

	1 h or overnight	1:1 LR-white in Ethanol
2 times	1 h	LR-white
Polymerization	24 h	UV Chamber in cold room

Protocol: HUVECs on ITO, adapted from [3]

Cell culture

Clean slides		wash in ethanol, dry, glow discharge (to make surface hydrophilic to promote cell adhesion)
Sterilize	15 min	UV
Coat		1% gelatin in PBS (0.1 M, pH 7.4)

Trypsinize cells and seed directly onto ITO slides
Grow cells up to desired confluency, 37°C, 5% CO_2

Fixation

Fix	30 min	2,5% PFA and 0,25% GA in phosphate buffer

Immunofluorescence staining

Wash 2 times		Tris–HCl buffer (0.05 M, pH 7.4)
Block	20 min	0.1% Triton X-100 and 5% Normal Goat Serum (NGS) in PBS
Primary	1 h	1:500 Rabbit anti Human VWF in PBS-5% NGS
Wash 2 times	5 min	PBS
Block	5min	PBS-5% NGS
Secondary	30 min	1:100 Goat anti-Rabbit Alexa 568 in PBS-5% NGS
Wash 2 times	5 min	PBS
Stain	20 min	1:20 Phalloidin Alexa 488 in PBS
Wash 3 times	5 min	PBS

Dehydration

2 quick washes	70% Ethanol
Dehydrate	5 min 70%, 5 min 80%, 5 min 90%, 5 min 100%, 15 min 100%, 15 min 100% Ethanol

Air dry slides

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