The formation of the immunological synapse between T cells and antigen-presenting cells (APC) begins within minutes of contact and can take hours for full T-cell activation. Although early phases of the synapse have been extensively studied for a select number of proteins, later phases have not yet been examined in detail. We studied the signaling network in stable synapses by measuring the simultaneous localization of 25 signaling and structural molecules over 2 h at the level of individual synapses using multi-epitope ligand cartography (MELC). Signaling proteins including phospho(p)ZAP70, pSLP76, pCD3ζ, and pLAT, along with proteins that influence synapse structure such as F-actin, tubulin, CD45, and ICAM-1, were localized in images of synapses and revealed the multi-dimensional construction of a mature synapse. The construction of the stable synapse included intense early TCR signaling, a phase of recruitment of structural proteins, and a sustained increase in signaling molecules and co-localization of TCR and pLAT signaling clusters in the center of the synapse. Consolidation of TCR and associated proteins resulted in formation of a small number of discrete synaptic microclusters. Development of synapses and cSMAC composition was greatly affected by the absence of Vav1, with an associated loss in PLCγ1 recruitment, pSLP76, and increased CXCR4. Together, these data demonstrate the use of multi-epitope ligand cartography to quantitatively analyze synapse formation and reveal successive recruitment of structural and signaling proteins and sustained phosphorylation at the mature synapse. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.025205, 2551–2567, 2013.

Adaptive immune responses are initiated by the meeting of a T cell and an antigen-presenting cell (APC)1 bearing peptide-MHC (pMHC) complexes that are a specific fit for the T-cell receptor (TCR) on the T-cell surface. Within seconds, TCR signaling starts with a sequence of phosphorylation and de-phosphorylation events of membrane-proximal and -distal TCR-signaling molecules and their spatial reorganization into protein multiclusters (1). Together with the rearrangement of structural molecules at the cell–cell interface, these signals lead to the formation of a supramolecular structure termed the immunological synapse (1–3). The synapse can differ substantially in size and composition, but comprises several common structural motifs (4–6). In the classical synapse, these structural motifs are organized in domains that form a target...
pattern. Two signaling areas form the middle of the synapse: the bullseye in the center is the central supramolecular activation cluster (cSMAC), dominated by TCR and associated signaling molecules, and the ring around it is called the peripheral (p)SMAC, dominated by the presence of stabilizing integrins (1, 6). The outermost ring, the distal (d)SMAC, is composed of F-actin important for structural integrity of the synapse. The purpose of the synapse, and its dynamic precursor the kinapse, is to translate information obtained from the APC on amount and quality of peptide and presence of coreceptors into T-cell actions such as proliferation or secretion (1).

TCR triggering activates a cascade of signaling events. First, Src kinases such as LCK are activated and phosphorylate the TCR-complexed CD3ζ chain on intracellular tyrosine-based activation motifs (ITAMs). Next, ZAP70 is recruited to ITAMs and phosphorylates adaptors such as LAT and SLP76. These in turn recruit PLCγ1, an activator of calcium flux, and Vav1, a regulator of actin reorganization, leading to activation of MAPKs and transcription factors such as NFAT and NF-κB (7, 8). The early TCR signaling leading to calcium flux takes place in seconds, followed by the appearance of the synapse within minutes. Recruitment and assembly of preformed complexes of signaling proteins facilitate structural formation of synapse and TCR signal amplification (9–11). For example, TCR and LAT are found in separate protein islands in the membrane, or in subsynaptic vesicles, and are brought together at the membrane to initiate signaling in microclusters (12–15). Microclusters are small aggregates of signaling proteins, adaptors, and TCR that change location in the synapse over time. Microclusters originate in the dSMAC and migrate in an actin-dependent manner through the pSMAC toward the center of the cSMAC (16, 17).

Current views on the formation and functions of the cSMAC are evolving with ongoing research. Because the cSMAC contains an accumulation of TCR, it was originally posited to serve as a platform for TCR signaling (2, 3). However, further investigations revealed that the centrally located TCR are not signaling-active and are down-regulated for recycling or degradation (17–20). The cSMAC is not uniform in composition but contains at least two different zones: a central zone where TCR signaling terminates, encompassed by a zone enriched with actively-signaling TCR, F-actin and associated coreceptors and kinases (9–12, 16, 21). It was proposed that the cSMAC modulates receptor degradation and signaling by regulating association of TCR with phospho-tyrosine proteins (22, 23).

Multi-epitope ligand cartography (MELC) is a microscopy-based technology that was developed to study the simultaneous locations of dozens of proteins in a single sample of fixed cells or tissue (24). The MELC system consists of a microscope, a CCD camera and a robotic transfer device enabling an automated sequence of delivering washing fluid and staining solution to the sample—all controlled by a computer. To perform MELC, the sample is stained with a fluorescent reagent, imaged, photo-bleached to completely remove fluorescence, washed, and then stained again with a subsequent fluorescent reagent until all detection reagents have been applied in sequence. The image data can be analyzed directly as raw fluorescence intensity or converted to binary data for each pixel confirming presence or absence of fluorescence for each protein. This resulting mathematical data can then be used to analyze colocalizations of all the proteins studied in the sample at a network-level scale (24–27).

We report here the results of using MELC technology to study the co-localizations of 25 molecules (24 proteins and DNA) in T cell–APC conjugates that were imaged at different time points to monitor synapse formation. To investigate the roles of these molecules in synapse formation for longer than the few minutes it takes to form the outlines of the cSMAC, cells were imaged over 2 h. MELC revealed a layered construction of the synapse with sequential recruitment of structural proteins and signaling molecules and a consolidation of LAT and TCR complexes that ultimately formed the mature synapse at late time points (60 min). Synapses contained a limited number of microclusters consisting of TCR associated with phosphorylated CD3ζ and signaling proteins in the cSMAC. Furthermore, MELC was used to analyze synapse formation in T cells from Vav1 knockout mice, and identified key differences in synapse signaling in the mutant T cells. Our results show that MELC technology is valuable in analyzing the large-scale translocation of many proteins in individual T cell–APC contacts simultaneously and thus can deliver new insights into the function and dynamics of formation of the immunological synapse.

**EXPERIMENTAL PROCEDURES**

**Mice**—OT-II mice carrying a transgenic TCR recognizing peptide of chicken ovalbumin (AA 323–339) peptide, pOVA, in the context of I-Ak have been described (28) and were used as a source of T cells. C57Bl/6 (H-2b) mice (from Harlan Germany) were used as a B cell source. Vav1−/− mice (29) were provided by Dr. Klaus-Dieter Fischer, University of Magdeburg, Germany. Animals were housed under specific pathogen-free conditions and treated according to institutional guidelines. All animal experiments were approved by the animal protection committee of the local authorities.

**Cell Preparation, Pair Formation**—Cell preparation and induction of B cell-T cell (BT) pair formation were performed as previously described (5). In brief, naïve CD4+ T cells from spleens of OT-II mice were enriched to over 90% purity (as assessed by expression of Vα2 TCR transgene) by negative isolation via immunomagnetic depletion (Miltenyi, Bergisch Gladbach, Germany). Naïve splenic B cells from C57Bl/6 mice were obtained by the same procedure with purities between 90 and 95%. For T cell activation, B cells were loaded with 100 μg/ml pOVA for 24 h and washed. Freshly isolated T cells were then mixed in a 1:1 ratio with B cells and co-incubated in an RPMI-based, FCS-supplemented media (Invitrogen, Los Angeles, CA). To synchronize pair formation, cells were centrifuged shortly at 260 × g and incubated at 37 °C, 5% CO2. Cells were used for subsequent analysis at indicated time points.
Slide Preparation for MELC—T and B cell mixtures were fixed with 2% paraformaldehyde for 15 min and adhered to poly-L-lysine-coated glass slides. Two to three different cell-coated areas per slide (reflecting different time points of pair formation or different biological samples, e.g. KO versus WT) were created by applying single droplets of cell suspension. Slides were stored in PBS at 4 °C until imaged by MELC.

MELC—

Antibody Library—We established a MELC library of 24 fluorescence tags (plus propidium iodide) as listed in Table I. We performed a stepwise selection process to judge specificity and usability of each antibody in our system. In brief, we first chose antibody products and clones based on evidence of previous successful and specific usage, ideally by multiple methods, as documented in peer-reviewed literature (see Table I). We then tested the performance of their directly immunofluorescent dye-coupled derivatives in our system by assessing the spatial distribution of the resulting signals (T cell-/B cell-specific, synaptic enrichment) as well as the signal quality (signal-to-noise ratio; details in Table I and supplemental Fig. S3). Most of the antibodies tested performed well in our system. However, several molecules of interest to the study of immune synapses (such as LFA-1) could not be included in the library as the signals delivered by the antibodies were not reliable, too weak or too unspecific in our system. Such, in the case of CD11a (LFA-1), the antibody did not deliver a T-cell specific pattern or synaptic enrichment in our hands. This was possibly due to effects of fixation and/or permeabilization in our protocol, which for technical reasons had to be identical for all markers. Wherever possible such molecules were replaced with stains for surrogate markers, e.g. the stain for LFA-1 was replaced by a stain for its main ligand ICAM-1, CD54. The appropriate working dilutions, incubation times and positions within the MELC run were worked-out in a series of pilot experiments based on previous conditions found to be generally suitable to MELC (24) and adapted to our system. During build-up of the MELC library, MELC runs were performed with varying and cumulative numbers and positions of the individual markers to ensure consistency of fluorescence signals in our system.

Image Recording by Toponome Imaging Cycler (TIC)—The sample was placed on the stage of an inverted wide-field fluorescence microscope (Leica DM IRE2; 63 × oil lens NA 1.40). For each of the two or three conditions defined by application of individual droplets of cell solution, one or two suitable fields of view were defined manually, and the corresponding XYZ-positions and a transmitted light reference image were acquired. A fully automated cyclic robotic process started using the spatial distribution of the resulting signals (T cell-/B cell-specific, synaptic enrichment) as well as the signal quality (signal-to-noise ratio; details in Table I and supplemental Fig. S3). Most of the antibodies tested performed well in our system. However, several molecules of interest to the study of immune synapses (such as LFA-1) could not be included in the library as the signals delivered by the antibodies were not reliable, too weak or too unspecific in our system. Such, in the case of CD11a (LFA-1), the antibody did not deliver a T-cell specific pattern or synaptic enrichment in our hands. This was possibly due to effects of fixation and/or permeabilization in our protocol, which for technical reasons had to be identical for all markers. Wherever possible such molecules were replaced with stains for surrogate markers, e.g. the stain for LFA-1 was replaced by a stain for its main ligand ICAM-1, CD54. The appropriate working dilutions, incubation times and positions within the MELC run were worked-out in a series of pilot experiments based on previous conditions found to be generally suitable to MELC (24) and adapted to our system. During build-up of the MELC library, MELC runs were performed with varying and cumulative numbers and positions of the individual markers to ensure consistency of fluorescence signals in our system.

Flow Cytometry—Antibodies against surface markers (CD4, CD19) were from BD Pharmingen, San José, CA, USA and antibodies for intracellular staining of total tyrosine-phosphorylated signaling molecules (anti-pTyr-Alexa488, clone PY20) were from Biolegend, San Diego, CA, USA. The integrin inhibitor BIRBT377 was a kind gift from Terence Kelly (Boehringer Ingelheim). Staining procedure was performed using the BD Fix&Perm Kit as indicated by manufacturer. Flow cytometry was performed at a BD Fortessa. BT pair formation was quantified as the percentage of all live OD4 + T cells in contact with B cells (CD19). Median fluorescence intensity (MFI) of signaling proteins was calculated and the signal from the identically labeled isotype control subtracted.

Western Blot—For analysis of pERK in cell pairs: co-incubation of T and B cells was stopped at 30 min. To some samples, anti-CD3 (2C11, 5 μg/ml) was added to boost signal. Cells were lysed as described previously (30) to obtain cytoplasmic extracts. Protein lysate (20 μg) was separated on a 10% SDS-PAGE and transferred to PVDF membranes. Proteins were detected with primary antibodies to phospho-ERK1/2 (S202/Y204, Cell Signaling) and beta-actin (Sigma), followed by HRP-coupled anti-rabbit and anti-rat secondary antibodies (BD Pharmingen) respectively, and analyzed with the Rotilumin detection system (Roth, Karlsruhe, Germany).

For ERK, Vav1, and SLP76 expression in WT and Vav1 KO cells were separated on a 10% SDS-PAGE and transferred to PVDF membranes. Proteins were detected with primary antibodies to phospho-ERK1/2 (S202/Y204, Cell Signaling) and beta-actin (Sigma), followed by HRP-coupled anti-rabbit and anti-rat secondary antibodies (BD Pharmingen) respectively, and analyzed with the Rotilumin detection system (Roth, Karlsruhe, Germany).

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**Statistical Analysis**—Student’s *t* test or a nonparametrical test where indicated in the results section were applied to assess statistical significance. Significance levels and symbols employed were *p* < 0.05 (*), *p* < 0.01 (**), and *p* < 0.001 (***). Statistical analysis and plotting was done with GraphPad Prism 5.01 (Graphpad Software, La Jolla, CA, USA) or MATLAB (The Mathworks Inc., Natick, MA, USA).

**RESULTS**

**Establishment of the MELC System to Study the Immunological Synapse**—The MELC system displays the location of many proteins simultaneously in fixed cells. This large-scale mapping of proteins makes it possible to do statistical analysis of protein co-localizations in subdomains of a cell. Here we used MELC to study the formation of the immunological synapse over a 2 h time course by following 25 molecules (24 proteins and DNA) in BT pairs. Murine OT-II TCR transgenic CD4+ naïve T cells (28) were stimulated with naïve primary B lymphocytes from C57/B6 mice as APC. B cells were loaded with saturating concentrations (100 µg/ml, ~105 nM) of a full agonist peptide (pOVA, AA 323–339, the cognate ligand to the OT-II TCR) (5). T cells and B cells were co-incubated, centrifuged to initiate conjugate formation, plated, and then fixed and permeabilized at various time points. The MELC system was programmed to subject the T cell–B cell pairs to 25 rounds of an automated sequence of staining and image collection, followed by bleaching of the fluorescence. The resulting microscopic images were then processed with algorithms to compensate for systemic artifacts such as lateral shift or residual, nonspecific fluorescence signals (24).

A representative field of view for BT pairs with stainings for 5 proteins (CD3ε, CD45R, pLCK, pLAT, pSLP76) is shown in Fig. 1A, with insets of magnifications of 4 selected pairs (labeled I-IV). Serial stainings of a BT pair show the cumulative appearance of fluorescence with pseudocolor applied to each stain to distinguish between the molecules (Fig. 1B). To confirm that the signaling protein localizations studied were specific to the APC – T cell synapse, we used T cell–T cell pairs as controls (Fig. 1C). In the upper block showing BT pairs and the lower block showing T cell–T cell pairs, F-actin, tubulin and DNA were similarly stained in both B cells and T cells. T cell marker CD3ε, total (t)-LCK, and CD45 stained T cells only whereas CD45R stained specifically B cells. Importantly, punctate staining of pLAT, pLCK, pSLP76, and pZAP70 was specific to the interface of the BT pairs. Further confirmation of the restriction of signaling to specific antigen-loaded BT pairs was provided by Western blot of activated ERK MAPK and by FACS analysis of phospho-tyrosine (supplemental Fig. S1A and S1B). The final library of antibodies and stains to target 25 molecules simultaneously is shown in Table I. A complete sequence of all 25 individual fluorescence signals within one representative BT pair is shown in supplemental Fig. S2.

We first compared the cell-type specificities and strengths of the 25 signals generated with the panel of antibodies and stains. We recorded the fluorescence signal intensities for each reagent stain at 30 min after conjugation in B cells alone, T cells alone, and T cell–B cell pairs. The resulting numerical values of signal intensity were displayed graphically (Fig. 1D, supplemental Fig. S3). The cell-type and BT-pair-specific expression pattern was statistically distinct for the majority of molecules and cellular entities. Several markers such as the co-stimulatory protein CD86, the integrin-ligand CD54, the chemokine receptor CXCR4 and phosphorylated signaling molecules such as pPLCγ-1 were represented by very weak or undefined signals when referenced to the total cell bodies. However, enrichment of these markers at the cell-cell interface forming the APC–T-cell synapse could be clearly visualized. Thus, measurements within the synaptic subregion of the cell pairs (as employed in Figs. 2f) resulted in statistically distinct and specific patterns for all 25 molecules involved in TCR signaling simultaneously at the level of individual BT pairs.

**Sustained Antigen-specific TCR Signaling During a Two-hour Process of Physical Maturation of the Synapses**—TCR activation triggers an immediate burst of signaling in TCR microclusters and subsequent organization of SMACs into central and peripheral zones within minutes. Subsequently, productive signaling through the T-cell synapse can last for hours. To determine the optimal time window for synapse formation, we evaluated pair formation and associated synaptic signaling in BT pairs in our system.

To more specifically allocate the signals detected in the BT pairs, we defined synaptic subregion based on the well-established division into central and peripheral signaling clusters (Fig. 2A–2C). The p- and cSMAC borders are generally defined by the presence of specific molecules such as LFA-1 or TCR respectively (2, 31, 32), but we chose instead to use spatial orientation and the location of pLAT to define these domains in the x-, y- and z-planes. pLAT was chosen as a marker for the center of the synapse and to define the ideal z-plane because pLAT was consistently found at the BT interface from 5 min after pair formation to the last time point and was typically found at the center of the synapse in a small number of discrete points. The F-actin ring around the synapse in the dSMAC was used to define the outer border of the pSMAC. We then developed an algorithm to use these F-actin and pLAT coordinates to calculate the position and alignment of the synapse in the x, y and z axes. The areas encircled by the actin ring ranged in diameter between 4 and 5 µm, and we defined the inner third of the areas as the cSMAC (Fig. 2A–C, Fig. S4).

Using this spatial synaptic definition we followed the kinetics of localization of key synaptic molecules within the pSMAC and cSMAC, respectively, in MELC. We observed (supplemental Fig. S1C) a gradual accumulation of F-actin in the peripheral subregion of the synapse with a peak at 60 min followed by a plateau. Similarly, CD3ε, an integral component
Fig. 1. Establishment of the MELC system. Sequential imaging of 25 molecules in synapse formation during in vitro activation of naïve CD4+ T-cells (OT-II) with specific antigen (100 μg/ml pOVA peptide)-loaded primary B cells as APC. A. Representative field of view in MELC imaging. Overlay image of the field of view acquired in individual fluorescence channels generated from 5 channels: CD3ε: red; CD45R: green; pLCK: pink; pLAT: cyan; pSLP76: yellow; PI (propidium iodide, nuclear marker): purple. Magnified insets (I-IV) demonstrate typical T-cell-B cell (BT) pairs. Scale bar in insets: 10 μm. B. Magnified, cumulative, multicolor overlay image of selected individual signals in one specific BT-cell pair with a characteristic synapse. For visual clarity, the CD45 signal is only depicted in the first and in the last two images. Scale bar: 10 μm. C, Phospho-signaling proteins at the synapse are limited to antigen-specific BT pairs. Upper panels: BT pair displaying a characteristic synapse. Top row: F-actin, tubulin, PI, phase contrast image of cell pair. Middle row: CD3ε, pLCK, CD45, CD45R, to show T or B cells. Bottom row: synapse-localized pLAT, pLCK, pSLP76 and pZAP70 signals. Lower block: Absence of signals in T-cell-T-cell pairs. Scale bar: 10 μm. D, Comparison of levels of fluorescence obtained from all 25 markers in BT pairs (BT), single B (sB) and single T-cells (sT). Intensity of raw fluorescent signals at the 30 min time point is shown was converted to a color scale to visually illustrate cell type-specific expression. Statistically significant expression (p < 0.05 or lower) is indicated by bars. Note that the raw intensity approach here fails to resolve small differences in expression for several markers demonstrating medium to very low intensity (CD86, CD54, CD3ε[Y1]). This formed the basis for the subsequent use of BT (synaptic) subregions and a pixel-based analysis (Figs 2ff). Analysis was performed in 32 BT pairs, 40 single B cells and 40 single T-cells in four independent experiments.
### Methods/Lit:
Key publications are listed where the actual product or the exact clone was employed by the methods indicated. Reports on the biology of the target protein with only indirect, not clone-specific evidence for antibody specificity were termed accordingly (Biology). More details can be found on the manufacturer's website. IB, Immunoblot; FC, Flow Cytometry; IHC, Immunohistochemistry; Biol., Biology.

### Distribution:
Predominant spatial distribution of the molecule based on the mean fluorescence intensity in the respective compartment: T cell (if single T [sT] > single B [sB]; t test, see all individual graphs and statistics in Fig. S3); B cell (if sT < sB); T and B (if sT not > sB and sT not < sB); Synapse (if Synapse [cS, central Synapse, cSMAC] > sT and sT ≤ sB).

### S/N Ratio:
Signal-to-Noise Ratio: Mean (mean fluorescence intensity in the respective compartment); S.D. (standard deviation); p, p value in the G test likelihood ratio to differ from an arbitrary threshold of 1.5.

| Molecule | Phospho site | Clone | Fluorophore | Source | Cat. no | Methods/Lit | Distribution | S/N Ratio (mean, S.D., p) |
|----------|--------------|-------|-------------|--------|---------|-------------|--------------|--------------------------|
| IA-IE (I-A/I-E) | 2G9 | FITC | BD Pharmingen | 553623 | IF, IB | B cell | 6,4360, 1,2158 | 0,000 |
| CD80 | 16-10A1 | Alexa488 | Biolegend | 104716 | IB, IHC, FC | Synapse | 1,6733, 0,0961 | 0,001 |
| CD86 | GL-1 | Alexa488 | Biolegend | 105018 | IB, IHC | Synapse | 2,7400, 1,0622 | 0,000 |
| CD54 (ICAM-1) | YN1/1.7.4 | FITC | Biolegend | 116112 | IHC | Synapse | 3,3510, 0,8439 | 0,000 |
| CD45R | RA3-682 | Alexa488 | BD Pharmingen | 557669 | FC, IF, IB | B cell | 7,0420, 1,9092 | 0,000 |
| CD4 | 30-F11 | FITC | BD Pharmingen | 553080 | FC, IHC, IB | Synapse | 5,9470, 0,6657 | 0,000 |
| F-actin | C4 | Alexa488 | BD Pharmingen | 558623 | IF, IB, FC | Synapse | 8,3430, 0,3413 | 0,000 |
| α-Tubulin | TU-01 | FITC | BD Pharmingen | 553753 | IB, IHC | Synapse | 2,8340, 0,3413 | 0,000 |
| CD184 (CXCR4) | 2B11 | FITC | BD Pharmingen | 551967 | FC, IHC, IF | Synapse | 1,6210, 0,1753 | 0,015 |
| CD4 | RM4-5 | Alexa488 | Biolegend | 557667 | FC, IHC, IF | T cell | 3,6280, 0,4174 | 0,000 |
| CD3ε | 17A2 | Alexa488 | Biolegend | 100210 | FC, IHC, IF | T cell | 6,7010, 1,0455 | 0,000 |
| pCD3ε [Y5] | ITAM 5, pY142 | K25-407.69 | Alexa488 | 558486 | FC, IB | T cell | 2,8600, 0,4193 | 0,000 |
| pCD3ε [Y1] | ITAM 1 | EM-26 | Alexa488 | SYBILLA* | Synapse | 1,5650, 0,5054 | 0,000 |
| tLCK | MOL-171 | Alexa488 | BD Pharmingen | 558504 | IB, IHC | T cell | 3,5660, 0,3203 | 0,000 |
| pLCK | p505 | 4 | Alexa488 | 557879 | Biol, FC | Synapse | 8,3430, 0,6657 | 0,000 |
| pLAT | p171 | 158-1169 | Alexa488 | 558519 | Biol, FC | Synapse | 2,9040, 0,5116 | 0,000 |
| tPLCγ1 | 10 | Alexa488 | BD Pharmingen | 558566 | Biol, FC | T cell | 2,2600, 0,1151 | 0,000 |
| pPLCγ1 | pY783 | 27 | Alexa488 | 557884 | Biol, FC | Synapse | 1,8160, 0,2890 | 0,000 |
| tSLP76 | H3 | Alexa488 | BD Pharmingen | 560056 | IB, FC | T cell | 3,2830, 0,2939 | 0,000 |
| pSLP76 | pY128 | J141 | Alexa488 | 558439 | Biol, FC, IHC | Synapse | 3,3450, 0,6042 | 0,000 |
| pZap70/Syk | pY319/pY352 | 17A | Alexa488 | 557818 | Biol, FC | Synapse | 1,7820, 0,2446 | 0,000 |
| tERK1/2 | G263-7 | Alexa488 | BD Pharmingen | 612592 | IB, FC | T and B | 5,3420, 0,5224 | 0,000 |
| pERK1/2 | pT202/pY204 | 20A | Alexa488 | 612594 | Biol, FC | T cell | 2,5110, 0,3598 | 0,000 |
| p-p38 | pT180/pY182 | 36 | Alexa488 | 612594 | Biol, FC | T cell | 1,5533, 0,0306 | 0,000 |
| PI (propidium iodide, nuclear marker) | | | Sigma | P4170 | Nucleus | 2,7450, 0,4333 | 0,000 |

* The pCD3ε [Y1] antibody was generously provided by Dr. J. Lindquist and the SYBILLA (Systems Biology of T-cell activation in Health and Disease) consortium.
of the TCR-CD3 complex, continuously accumulated and reached high levels at 120 min for most of the synapses in the population. As CD3 accumulation in the cSMAC is considered to represent structural maturation of the synapse, we concluded that the physical maturation of the synapse in our system was completed after 120 min. In addition, we observed pLAT to be consistently enriched at the cSMAC throughout the entire observation period, which suggested ongoing signaling activity. Likewise, using conventional flow cytometry measuring signals of an anti-phospho-tyrosine monoclonal antibody (PY20) we observed high signaling activity in APC-T cell pairs exceeding the levels found in single T cells at early (5 min) and late (120 min) time points (supplemental Fig. S1D). Of note, onset of pair formation was immediate after the synchronized APC-T-cell contact. Most BT pairs had formed already after 5 min and were strictly dependent on the presence of specific antigen and integrity of integrins (supplemental Fig. S1E).

Based on these kinetic evaluations, we decided to use MELC to study T-cell signaling events at various time points.
Distinct Phases of Molecular Recruitment and Colocalization Define Kinetic Changes in the Signaling Signature of the Synapse and Reveal a Delayed “Signaling Maturation”—Given the known centripetal movement of TCR microclusters through the pSMAC to the cSMAC (12, 40), we sought to analyze the location frequencies of our set of TCR signaling proteins in these individual compartments. As TCR signaling efficiency relies on coordinated spatial arrangement of a network of molecules we also aimed at looking into specific colocalizations of signaling proteins in the stable synapse.

As the fluorescent signals obtained for phosphorylated signaling proteins at the synapse were relatively low (Fig. 1D) we applied a threshold algorithm that transformed the 16-bit intensity signal into binary information for each protein at a pixel: either present (1) or absent (0) (supplemental Fig. S2). This approach also facilitated collection of signals of phospho-proteins from small subregions of the cells that measured only a few pixels in diameter. The amount of pixels positive for each signal within a subregion was then recorded and compared with the total number of pixels in this subregion to obtain the relative frequency of each protein in a given compartment of the synapse.

Using this approach, we analyzed the location of individual signaling proteins in the subcompartments of the immune synapse with MELC. Characteristic changes are illustrated as an archetypal graphical display of synapse formation in Fig. 3A (complete list in supplemental Table S1). The analysis revealed several layers in the synaptic buildup based on the consecutive recruitment of groups of proteins.

First, the kinetics of signaling in the synapse showed the previously mentioned high signaling levels at 5 min and subsequent decline at 10 min likely reflecting a period of resetting following the forced onset of pair formation. The decline of active signaling molecules toward 10 min in the cSMAC was significant for pZAP70 but pSLP76, pLAT, pLCK, pCD3ζ[Y5] and pCD3ζ’[Y1] also showed a decline (supplemental Table S1). In contrast, other molecules like F-actin showed no decrease but rather increased toward 10 min.

Second, between 10 and 30 min key components of TCR signaling including pZAP70, pSLP76, and pPLCγ1 increased strongly and remained high from then on. These dynamic changes likely reflected consistent TCR signaling via the now evolving structurally stable synapse following the resetting and reorientation phase.

Third, between 30 and 60 min, the data showed a rise in the recruitment of molecules supportive for TCR signaling such as tERK in the cSMAC and the chemokine receptor CXCR4 in the pSMAC. This suggested an increased build-up of the physical structure and binding strength of the T-cell–B-cell pair during
**FIG. 3.** Distinct phases of molecular recruitment and co-localization in pSMAC and cSMAC during buildup of the mature synapse. 

**A,** Sequential molecular assembly of the synapse with phases of early TCR signaling, structural enforcement and delayed accumulation of signaling molecules. Data obtained in MELC. Characteristic changes in positioning and amount of molecules are indicated by symbols. A complete list of numerical data is given in supplemental Table S1. **B,** Localization of selected signaling molecules pLAT, pCD3ζ[Y5], pZAP70, and pSLP76 in synaptic subcompartments during stepwise formation of the stable synapse between 10 and 120 min. Graphical Tables illustrate the relative frequencies of pixels positive for the indicated proteins over time in the cSMAC and pSMAC subcompartment. A color-coded scale in shades of orange enables a visual scan of data to detect trends: Color shades and numbers in the orange-colored boxes represent relative frequencies of a protein (0.0, minimum, yellow; 1.0, maximum, orange) relative to minimum and maximum frequencies (% of positive pixels in compartment) of that protein (white boxes, Rel. Freq.: Min, Max). Green squares identify protein represented by row. Visualized is a continuous increase of signaling molecules in the cSMAC toward 60 to 120 min (identical trend in pSMAC). Statistical significance is shown using bars (thin line, \(p < 0.05\); thick line, \(p < 0.01\)). **C,** Co-localization of signaling molecules confined to synaptic areas positive for pLAT- and CD3ζ[Y5]. Graphical Tables illustrate CMPs and their relative frequencies over time using the orange color-coded scale. The increase in signaling molecules in cSMAC appears more pronounced (16-fold for areas with co-localizing pLAT, CD3ζ, pZAP70, and pSLP76) than for single molecules alone. Bars indicate statistical significances (thin line: 0.05; thick line: \(p < 0.01\)). **D,** Opposed kinetics of co-localization.
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this period. Supporting this, we previously described an increase in binding forces between T cell–APC conjugates after 30 min (41). In addition, the presence of the CD45 phosphatase and its splicing form CD45R in the pSMAC decreased between 30 and 60 min of synapse formation. These changes all suggested a critical phase of molecular recruitment favoring binding and prolonged TCR signaling.

Finally, between 60 and 120 min, we observed a continuous rise of active signaling molecules such as pCD3ζ and pZAP70 in the cSMAC and recruitment of tPLCsγ1 toward the center and of F-actin toward the periphery of the synapse. These events occurred at timepoints accompanying the final structural maturation of the synaptic population studied (Fig. 2) and indicated a delayed TCR signal amplification and change in TCR signaling signature.

Next, to analyze the kinetics and distribution of TCR signaling in more depth, we focused on selected signaling proteins and their colocalizations during the period of the proper formation of the stable synapse in our system, i.e. between 10 and 120 min. We initially analyzed the presence of pLAT, pCD3ζ(Y5), pZAP70, and pSLP76, in cSMAC and pSMAC, respectively (Fig 3B). We found that all three molecules displayed a gradual increase of the frequencies of these individual molecules in both cSMAC and pSMAC (between 1.4-fold, pSLP76 in cSMAC; and 3.5-fold, pZAP70 in cSMAC). Highest levels were reached at 60 and 120 min, respectively, suggesting ongoing recruitment of signaling molecules at delayed time points.

We next wanted to analyze the frequencies of co-localizations of these synaptic molecules (Fig. 3C). We tested the frequencies of co-localization of pZAP70 and pSLP76 in synaptic areas that were also positive for pLAT and CD3 (pCD3ζ(Y5)). The use of binarized imaging data resulting from the thresholding of fluorescent signals was not only useful in evaluating low-intensity signals of individual markers, but could also be used to generate and quantify CMP motifs (25, 42, 43), patterns of localizations of several markers within one spot (pixel). Although it is conceptually easier to think of a CMP motif as a protein complex, MELC analysis does not provide data on actual binding between proteins—only on shared locations. Using such colocalization CMPs for analysis, the accumulation of signaling molecules in the cSMAC over time became more obvious. For example, the frequency of pixels positive for pLAT, pCD3ζ(Y5), and pZAP70 increased 9.5-fold (from 2.5% to 23.8%) and the frequency of colocalizations of pLAT, pCD3ζ(Y5), pZAP70, and pSLP76 increased 16.2-fold (from 1.0% to 16.2%) (Fig. 3C).

Finally, we studied the frequency of synaptic areas distinctively characterized by the presence or absence of pLAT and CD3ζ, respectively (Fig. 3D). pLAT is considered to mainly correspond to membrane domains or vesicles, and CD3ζ primarily localizes with TCR in domains separate from those containing pLAT (12). During the course of synapse formation, we found that the frequencies of CMPs containing pLAT but not pCD3ζ peaked very early in the pSMAC (at 10 min) and at 30 min in the cSMAC and then strongly decreased over time (in the pSMAC by approximately two-thirds, from 71.2% to 25.9%). In contrast, (TCR)-areas containing pCD3ζ but not pLAT showed an increase in both pSMAC and cSMAC peaking at 120 min (in the cSMAC from 8.2% to 20.3%). Both pCD3ζ+pLAT- areas and pCD3ζ+pLAT+ areas (Fig. 3C) were found at higher frequencies in the cSMAC than in the pSMAC (20.3 versus 11.9%, and 59.8 versus 34.2%, respectively). However, the area of the pSMAC was 8 times that of the cSMAC according to our definition of the cSMAC as 1/3 of the radius of the pSMAC. The continuous increase of CD3ζ was consistent with the CD3ζ enrichment observed earlier and paralleled the general recruitment toward the synapse (supplemental Fig. S1C). Our observation of an increase of pLAT clusters (Fig. 3B), and increase of pLAT+pCD3ζ+ (Fig. 3C) yet decrease of pLAT+pCD3ζ- (Fig. 3D) suggests that the TCR and pLAT clusters became confluent over time in both pSMAC and cSMAC. Indeed, the merging of LAT with TCR/CD3 clusters during T-cell activation has been described to start within minutes after onset of synapse formation (14). Here we observe this process on a multiprotein level for longer time periods, up to 120 min.

In summary, these findings suggest that sustained TCR signaling within the synapse is accompanied by a gradual accumulation of distinct clusters of signaling molecules and a delayed change in signaling that we would call synaptic “signaling maturation.”

Molecular Composition of Microclusters at the Center of the Synapse—TCR signaling in T cells on planar lipid bilayers takes place in numerous microclusters that take between 70 and 140 nm (14) and vary from 10 to close to a 100 TCR signaling molecules per cluster while in transit to the cSMAC (44). Studies of T cell–APC conjugates immobilized in an upright dimension of signaling molecules in synaptic subcompartments with distinctive presence or absence of pLAT and CD3ζ(Y5). Green and red color code for the presence or absence of the protein markers indicated. pLAT cluster (in absence of CD3ζ) peak early in pSMAC (10 min) and then disappear. In contrast, signaling-associated pLAT cluster (pLAT+, CD3ζ−, ZAP70+, SLP76+) and CD3ζ-cluster increase toward 60 to 120 min in the cSMAC (identical trend in pSMAC). Bars indicate statistical significances (thin line: 0.05; thick line: p < 0.01). E, shows two of the characteristic CMP motifs illustrated (and highlighted by frame) in D as box plots. Bars indicate statistical significances (thin: 0.05; thick: p < 0.01). pLAT and CD3ζ were used as base motifs to define the subregion for analysis in C and D, respectively. CMP analysis of 130 synapses.
ализировано преимущественно в нескольких микрокластерах (~2–3 пикселей (600–900 нм) в диаметре). Эти микрокластеры окружали центр синапса и содержали активированные сигнальные молекулы, включая pSLP76, pLAT, pLCK, ILCK (Фиг. 4А). Мы использовали CMP-анализ, чтобы сравнить молекулярную композицию сигнальных микрокластеров, содержащих pSLP76+ области, с молекулярной композицией pSLP76− областей (Фиг. 4В). pSLP76 был выбран из области сигнальных молекул, где его сигнальная композиция показала высокий отношение сигнал/шум и ясно отразила расположение и форму сигнальных микрокластеров. CMP-анализ показал, что pSLP76 был сильным и значимым колокализованным с pLCK, pLAT, pCD3ζ, pZAP70, и PLCγ1. В отличие от этого, CD4, CD86, CD45, и общие сигнальные молекулы, такие как tPLC-γ1, оставались малыми или не имели тенденции к аккумуляции в этих микрокластерах. Также, Фактоин был найден более часто вне этих микрокластеров, чем в них (Фиг. 4С). Таким образом, сигнал в синапсе концентрируется в одном, микрокластеров активного TCR-сигнализации (включая pSLP76, pLAT, pLCK, pCD3ζ, pZAP70, и PLCγ1) в то время как окружающая область определяется более молекулами, которые обеспечивают структурность (F-актин) и сигнал терминации (таких как CD45) (Фиг. 4D).

**Altered Molecular Composition of Synapses in T Cells from Vav1−/− Mice** — На основании применения технологии MELC-анализа к исследованию синапса было бытовать использовать для характеристики сигнальных дефектов в T-лимфоцитах при использовании мышей с Vav1-дефицитом (Vav1−/−). Vav1 требуется для развития и активации T-лимфоцитов, используя сигналы приводящие к внутриклеточному кальциевому притоку, MAPK активации и актином кластеризации (46). Vav1 может функционировать как структурное белка, контролируя молекулярную композицию сигнальных областей, регулируя кальциевую сигнализацию (47). Vav1 также может быть рекрутирован и стабилизировать pSLP76 микрокластеров (48, 49). Чтобы исследовать молекулярную композицию синапсов в отсутствие Vav1, мы применяли технологию MELC-анализа у T-лимфоцитов Vav1−/− мышей.

**Fig. 4. Molecular composition of microclusters at the synapse.** A, Localization of pSLP76+ clusters around the center of the synapse. Micrographs of a representative BT-cell pair (first panel) show the cumulative overlay of images of structural molecules (F-actin, CD45, CD45R) with signaling molecules (pSLP76, pLAT, pLCK, ILCK) in the indicated colors (next 5 panels). B, En face view of a representative synapse at a B cell-T-cell pair. Original image stained for the same proteins as in (A) (left image) and 2 axial en face views (middle and right image) through the synapse showing all molecules (middle image) or signaling molecules only (right image). Note the presence of discrete microclusters around the cSMAC. Scale bar: 10 μm. C, Molecular composition of pSLP76+ clusters in the synapse at 60 min. CMP analysis shows percentage of pixels positive for the given protein colocalizing with pSLP76 (pSLP76+) compared with the percentage of pixels in areas (pixels) that do not contain pSLP76 (pSLP76−). These values are compared in the ratio column (pSLP76+/pSLP76−). Highest ratios are colored (red: ratio>4; orange: ratio>2). Significant p values are colored (green: p<0.05; blue: p<0.01). D, Graphical illustration of the composition of pSLP76+ clusters in the cSMAC and the surrounding area, respectively. Red bars point to the projected locations of the molecules listed. Potential functions of the respective clusters are indicated (signaling versus structure/signaling termination).
We first quantified the overall capacity of Vav1−/− T cells to stably bind to APC and to form synapses. The numbers of conjugates formed was strongly reduced in BT pairs with T cells from Vav1−/− mice (from 31.1 ± 4.9% for WT to 11.2 ± 5.2% for Vav1−/−) (Fig. 5A,5B). Similar data were obtained by flow cytometry (supplemental Fig. S5A). The overall signaling in Vav1−/− T cells was defective as shown conventionally by Western blot demonstrating greatly reduced activation of pERK in total cell extracts (supplemental Fig. S5B), however tSLP76 expression was normal in these cells (supplemental Fig. S5C). ERK and SLP76 were chosen because Vav1 is known to induce ERK activation (46) and to influence stability of the SLP76 complex (49). CMP analysis of individual synapses formed by Vav1−/− T cells revealed significant shifts in groups of proteins when compared with synapses by WT T cells (supplemental Table S2). First, in WT synapses (supplemental Table S2, Part A) TCR-associated molecules CD4, pCD3, and pCD3ζ were much more likely to be found in WT synapses than in Vav1−/− synapses. Second, structural and costimulatory proteins CD80/86, CXCR4 and F-actin were all present at lower frequencies in WT synapses than in Vav1−/− synapses. These differences are more obvious when directly looking at the CMP motifs found be higher in Vav1−/− synapses (Table S2, Part B). Third, several signaling molecules were mislocalized in Vav1−/− synapses. For example, CD45 was found enriched in Vav1−/− synapses suggesting failure in synaptic exclusion. Most notably, total PLCγ1 was greatly reduced in Vav1−/− synapses and consequently also pPLCγ1. Given the importance of Vav1 to SLP76 microclusters (49), we expected to find reductions in tSLP76 at the synapse but tSLP76 was unexpectedly less enriched in WT synapses. However, motifs containing pSLP76 were clearly expressed at lower frequencies in Vav1−/− synapses again suggesting inadequate signaling in Vav1−/− synapses. In contrast, no or only minor differences were found in localization of LCK, pLAT, pZAP70, CD54, p38 and ERK, indicating that
localization of these proteins does not primarily depend on Vav1. Images of selected CMP motifs and quantitative comparisons of their occurrence in WT and Vav1\(^{-/-}\) synapses are shown in Fig. 5C. A graphical summary of the signaling profile in the Vav1\(^{-/-}\) T cells illustrates these alterations in signaling clusters, showing the impaired recruitment of tPLC\(_{\gamma1}\), pPLC\(_{\gamma1}\) and pSLP76 (Fig. 5D). These data confirm that Vav1 regulates PLC\(_{\gamma1}\) localization, either directly or indirectly through protein complex formation, SLP76 activation, and reveal a novel defect in CXCR4 localization. These results represent the first large-scale imaging data on Vav1\(^{-/-}\) T-cell synapses and validate the use of MELC to detect and quantify molecular signaling defects at the level of the individual synapses.

**DISCUSSION**

Current imaging technologies directed at the study of the synapse are limited in the number of proteins that can be tracked simultaneously. MELC technology has been used to map multiple proteins in the same sample of fixed cells or tissues. In this report, we used MELC for the first time to study 25 molecules at the immune synapse over an extended period of time. We evaluated individual reagent stains over five time points and performed quantitative analysis of co-localizations of proteins and the relationships between different classes of proteins at the synapse. We used the resulting data to characterize the layered construction of the synapse, the composition of late-stage microclusters, and the structure of Vav1 mutant synapses.

Signaling at the synapse takes place in microclusters of proteins containing TCR, phosphorylated TCR-proximal signaling proteins including pLCK and pZAP70 and adapter proteins including pLAT and pSLP76. Studies on live T cells immobilized on lipid planar bilayers demonstrated that microclusters originate in the dSMAC and migrate in an actin-dependent manner to the center of the cSMAC (12, 40). We analyzed images of signaling changes over 2 h at the synapse of fixed cell pairs to identify trends in synapse formation. We used primary mouse naïve T cells and naïve B cells, a homogenous APC population that display lower levels of co-stimulatory and danger signals than macrophages and dendritic cells. With these cells, central localization of TCR clusters took between 30 and 60 min, and TCR signaling, as measured by the appearance of phosphorylated proteins, peaked at 60–120 min, consistent with previous observations (3, 17, 19). While individual signaling molecules have been followed at the synapse for over an hour (50), MELC enabled us to analyze network-scale numbers of signaling proteins in this time period.

Results from MELC revealed that the construction of the synapse occurred in overlapping layers of proteins classes. Based on recruitment of F-actin toward the periphery and of CD3 toward the center of the synapse we observed the structural maturation in our synaptic population to be complete after 120 min. Already at 5 min, we observed high levels of phosphorylated molecules suggesting a rapid burst of signaling from the sudden contact of T cell and B cells after centrifugation, along with an appearance of pLAT and pZAP70, but little F-actin or SLP76. This initial jolt of signaling tapered off by 10 min, probably as T cells re-oriented to build a stable synapse. The proper buildup of the mature synapse including formation of a cSMAC (central CD3\(_e\) accumulation) then started at 10 min when intense TCR signaling (re-) started and continued throughout. This was supported by processes of consolidation and structural reinforcement that ranged from 30 to 60 min. At this time, costimulatory proteins like CD86 and CXCR4 began to appear and nonactivated signaling proteins such as tERK were recruited. These events indicated a period of structural enforcement and increased binding strength of the cell-cell contact. In accordance with this, we have previously observed a rise in binding forces between APC-T-cell pairs after ~30 min of APC-T-cell contact (41). Furthermore, CD45R and CD45 were reduced in the synapse area likely resulting in a release of TCR signaling from phospha-tase inhibition, further indicating that a platform for signaling was being assembled. A last, protracted stage of strong TCR signaling accumulation and coalescence was apparent at the timepoints 60 min and 120 min. The overall levels of signaling molecules present in pSMAC and cSMAC further increased compared with earlier timepoints. Signals by pLAT in the pSMAC peaked at 60 min, although signaling continued to rise in the central synapse area toward 120 min. Thus, synapses were built in stages with a structural platform that accompanied a gradual increase in signaling by pCD3\(_\zeta\), pZAP70, pLAT, pSLP76 over the entire 2 h and a prolonged and gradual shift from pSMAC to cSMAC signaling.

To parse the numerical data derived from our MELC analysis of synapses, we compared CMPs containing pLAT and/or TCR (CD3\(_\zeta\)) over the time course of the study. pLAT was chosen as a marker of membrane domains that are initially distinct from TCR membrane domains. It has recently been shown that LAT is recruited to the membrane in sub-synaptic vesicles where it becomes phosphorylated at SLP76 microclusters, and that when LAT domains couple to TCR domains they become microclusters (12, 15). LAT and TCR are also located in separate protein islands that concatenate on activation (14). Analysis of CMP featuring pLAT alone versus CMP with TCR alone revealed that they exhibited dynamics consistent with the merging of LAT and TCR into clusters. As the CMP containing pLAT alone without TCR declined in the pSMAC, they increased in the cSMAC together with CMP containing both pLAT and TCR, suggestive of a merging of two different clusters to make a functional signaling TCR microcluster (14). In addition, the high frequency of colocalized signaling mediators at delayed time points underlines the potential importance of these late stages of synapse formation for T-cell activation. Indeed, the time point of 120
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min coincides with the minimum time needed for a B-cell–T-cell contact to ensure full T-cell activation (3).

We found that signaling within the synapse culminated in a small number of large microclusters at the border between the pSMAC and cSMAC. Within these microclusters the proteins most specifically co-localized were active pSLP76 (used to define the clusters), pLAT, pLCK, pZAP70, and pCD3ζ. This cluster composition corresponds to that described for proper microclusters (signalosomes consisting of CD3ζ, pZAP70, pSLP76, pLAT, pLCK, and others) and their surrounding area (consisting of CD3ε, pERK, CD45, and others) as previously reported (40). Surprisingly, F-actin was negatively associated with these microclusters. It is possible that these microclusters (rendered at 60 min onset of pair formation) represent late-stage signaling in a mature, fully developed synapse. The large size of the microclusters, the small number of them, the location and the negative association with F-actin are all consistent with the possibility that these microclusters have coalesced, moved toward the center of the synapse and are detaching from actin, but can nonetheless still actively signal.

MELC was also used to analyze synapse formation in Vav1 mutant T cells. We observed that loss of Vav1 resulted in an approximate two-thirds decrease in the number of synapses formed, which represents a challenge in conventional biochemical analysis of signaling in synapses. However, MELC assessment of mass protein localization at single T-cell level could still point to defects in Vav1 KO T cells that might lead to insights into development of mutant cells. Given that Vav1 is mutated in the T-cell germline of the mice used here, we reasoned that it is possible that any signaling-active T-cells were selected during development for the ability to overcome the loss of Vav1, possibly by having greater levels of proteins that would make TCR signaling easier (51) and that MELC might be helpful in detecting them. For example, our CMP array showed that CXCR4 is significantly increased in the synapses of Vav1 T cells. CXCR4 is associated with TCR, recruited to synapses and signals through ZAP70 (52). It is possible that increased levels of CXCR4 at the synapse generate enough additional signaling through ZAP70 to partially compensate for lower levels of TCR and higher levels of CD45, leading to signaling-active Vav1 KO T cells. However, the severe decrease in PLCγ1 recruitment to the synapse we observed would hinder normal TCR signaling to calcium-dependent targets such as NF-kB or NFAT. In contrast, we observed normal localization and activation of the critical adaptor LAT, indicating that some of the main TCR signaling events can still occur without Vav1.

In the future, MELC results can be expanded by using new techniques such as using grids to hold conjugate pairs in place to enable clear imaging of the interface planes (45), or using super-resolution imaging technologies (53, 54) to improve resolution to low nm scales. We demonstrated here the use of MELC as a tool to analyze the architecture of the synapse over 2 h, the composition of microclusters, and the synapse composition following loss of a key TCR signaling protein, Vav1. This report highlights the advantages of using MELC to perform network-scale studies to shed light on the complex functions of the immune synapse.

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